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<b>(54) Title:</b> COMPLEX CATIONIC LIPIDS			
<b>(57) Abstract</b>  Cationic lipids, having a derivated quaternary ammonium head group, that provide improved cell targeting ability and enhanced transfective efficacy for negatively charged macromolecules. The lipids comprise a linker having functional groups that provide sites for further attachment of drugs, cell receptor ligands or other bioactive agents.			

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### COMPLEX CATIONIC LIPIDS

The present invention relates to cytofectins comprising complex amphiphilic lipids in which a bioactive agent which is recognized by biological systems is joined to a Rosenthal Inhibitor core structure through an alkyl linking group. A number of alkyl linking groups are disclosed, including carboxy, carbamyl, ureyl, thioureyl, and guanidyl cytofectins.

A second aspect of the present invention relates to such cytofectins having a primary amine within 8 carbons of the quaternary nitrogen.

A third aspect of the present invention relates to such cytofectins wherein the biologically active moiety is an amino acid or peptide selected from those moieties which are non-polar, polar and uncharged, and negatively charged at physiological pH.

A fourth aspect of the present invention relates to such cytofectins wherein the biologically active moiety contains alternative amino acids which are not generally found in natural organisms.

### BACKGROUND OF THE INVENTION

Cationic lipids are amphiphilic molecules having a lipophilic region, commonly comprising one or more hydrocarbon or alkyl groups, and a hydrophilic region comprising at least one positively charged polar head group. Cationic lipids are useful for facilitating the transport of macromolecules through the plasma membrane of cells and into the cytoplasm by forming net positively charged complexes. The process, which can be carried out *in vivo* as well as *in vitro*, is known as transfection, and the cationic lipids used in such techniques are known as cytofectins.

Cytofectins which enhance transfection efficiency as little as 3 fold over that observed with naked DNA are beneficial, although preferably transfection efficiency is increased 5-10 fold, and more preferably transfection efficiency is enhanced more than 10 fold.

Typically, cytofectins are combined with a neutral zwitterionic lipid such as a phospholipid, because it has been found that the two amphiphilic lipid species in combination are able to form vesicles comprising ordered lipid bilayers that are more effective at transfection than the cytofectin alone. These vesicles, or liposomes, have multiple positive charges on the surface which allow them to form a complex with a polynucleotide or other anionic molecule such as negatively charged proteins. Remaining net cationic charges on the surface of the polynucleotide/cytofectin/neutral lipid complex are capable of strong interaction with the predominately negative charge of the cell membrane surface.

Apart from the basic features of amphiphilic properties and the polar head group, cytofectins have considerable structural diversity in the lipophilic and hydrophilic regions. Many different cytofectin species have been synthesized for use in transfection and are now commercially available. Such cytofectins include, for example, Lipofectin™, Lipofectin ACE™, LipofectAMINE™, Transfectam™, and DOTAP™. The structural diversity of effective cytofectins reflects, in part, the observation that structure-function-recognition aspects of cytofectins differ with respect to distinct applications in cells. Experience with cytofectins structurally similar to the DOTMA compounds

indicates that transfection activity depends in part on the cell type transfected (Felgner et al. *J. Biol. Chem.* 84:7413-7417, 1987; Wheeler et al. *Biochem. Biophys. Acta*, in press). Particularly, cationic lipids comprising spermine substitution of the ammonium groups proved more effective than DOTMA for transfection of some cell lines. This phenomenon suggests that effective transfection depends not only on passive fusion of the cationic lipid complex with the structural lipid bilayer of the plasma membrane, but on specific cellular characteristics and interaction between cell components and the individual cationic lipid species.

Structural variants among cytofectin species are therefore an indication of a more sophisticated understanding of the multiple and complex interactions of cytofectins with cells, and an effort on the part of investigators to take advantage of one or more of these interactions.

DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium, disclosed in U.S. Patent No. 5,049,386 to Epstein, was one of the first cationic lipids developed, and lipids of this group have become reference compounds in evaluating comparative cytofectin potency in the development of new structural variants. DOTMA lipids are characterized by a propanaminium group having a quaternary nitrogen, which provides the cationic site of the molecule, together with a pair of C<sub>18</sub> hydrocarbons that are ether-linked to the propyl backbone of the molecule. The quaternary nitrogen is trisubstituted with relatively shorter alkyl chains, such as methyl groups. A structurally similar cationic lipid, 1,2-bis(oleoyloxy)-3-(3-(trimethylammonia)propyl)propane (DOTAP), comprises acyl, rather than ether-linked alkyl groups, and is believed to be more easily metabolized by target cells.

Some species of cationic lipids, for example, ammonium salts directly substituted by alkyl or acyl groups, were developed primarily for purposes of economy (U.S. Patent No. 5,279,833 to Rose). Others were developed in an effort to provide less toxic effects; for example, a highly biocompatible cytofectin prepared from phosphatidylcholine and sphingomyelin: 1, 2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (Avanti Polar Lipids, Inc. Alabaster, AL, Cat. Nos. 890700-706).

U.S. Patent No. 5,264,618 to Felgner et al., the contents of which are incorporated herein by reference, discloses cytofectins that are structurally similar to the Rosenthal Inhibitor (RI) of phospholipase A (Rosenthal et al., *J. Biol. Chem.* 235:2202-2206, 1960) and diacyl- or alkyl/acyl- species thereof. The RI based series of compounds are known by acronyms having the pattern: DORIE (C<sub>18</sub>); DPRIE (C<sub>16</sub>); and DMRIE (C<sub>14</sub>). These acronyms imply a common basic chemical structure; for example, DMRIE is 1-propanaminium, N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-, bromide, (+) (CAS registry:146659); the others differ in their substituent alkyl groups. These cytofectins, having a polar hydroxyethyl substituent on the quaternary ammonium group, provide more effective transfection in many cases than DOTMA type compounds. A study of the effect of varying substituents at the hydroxyalkyl moiety and variation of alkyl chain lengths on the transfection efficacy of the RI cytofectins is presented in Felgner et al. (*J. Biol. Chem.* 269:2550-2561, 1994). Again, the studies showed that the optimum hydroxyl alkyl chain length is cell-type dependent.

The conversion of DMRIE to  $\beta$ AE-DMRIE (Wheeler et al., *Biochem. Biophys. Acta*, in press) has been found to have a significant effect on cytofectin activity. DMRIE, which has a quaternary nitrogen adjacent to a primary alcohol, thus imparting a pH independent positive charge, is one of the most active cytofectins now known.

However, the substitution of a primary amine group for the alcohol on DMRIE to give  $\beta$ AE-DMRIE was found to form DNA complexes that are structurally distinct from those with DMRIE, and  $\beta$ AE-DMRIE is able to transfect many cell lines effectively in the absence of helper co-lipids. The observation that a single substitution in the cytofectin skeleton can provide marked changes in transfection properties suggests that other modifications can bring about similar improvements in gene delivery.

Continuing studies of the transfection event indicate that cationic lipids may facilitate not only entry of the functional molecule into the cytoplasm of a cell, but may also provide additional beneficial capabilities; for example, protecting the functional molecule from lysosomal degradation, facilitating entry into the nuclear compartment, or even preventing the degradation of the RNA transcription product by cytoplasmic enzymes. These functions of cationic molecules are believed to be related to specific structural features. Accordingly, there is a need for cytofectins that are particularly suited to transfection of foreign molecules into specific cell types. There is also a need to develop cytofectins that are able to perform specific intracellular functions.

#### SUMMARY OF THE INVENTION

The present invention relates to a number of linkage groups for coupling a bioactive agent which is recognized by a metabolic system to a cytofectin having a Rosenthal Inhibitor core structure.

A second aspect of the present invention relates to such cytofectins having a primary amine within 8 carbons of the quaternary nitrogen.

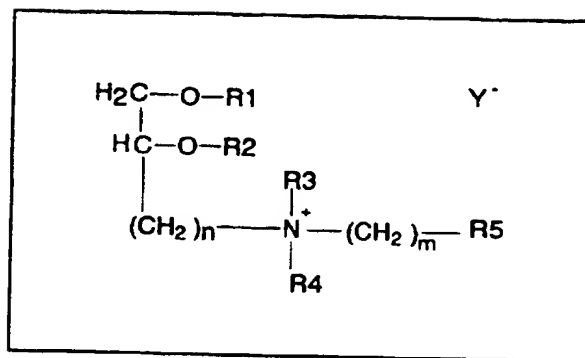
A third aspect of the present invention relates to such cytofectins linked to an amino acid or peptide selected from those moieties which are non-polar, polar and uncharged, and negatively charged at physiological pH. Examples of polar and uncharged amino acids are those possess a side chain having a polar moiety, such as serine and threonine. Examples of non-polar amino acids are those such as glycine, valine, phenylalanine, and leucine, which possess a linear alkyl or cyclic alkyl or heteroalkyl side chains. Examples of negatively charged amino acids are those such as glutamate or aspartate which possess a negatively charged acidic side chain.

A fourth aspect of the present invention relates to such cytofectins linked to an amino acid or peptide which contains alternative amino acids which are not generally found in natural organisms. Such amino acids include but are not limited to the modified and unusual amino acids listed in 37 C.F.R. § 1.822. In addition, such amino acids may be synthetic amino acids not found in nature.

One embodiment of the present invention is a cationic amphiphilic lipid comprising:

- a Rosenthal inhibitor core structure comprising a quaternary nitrogen group;
- an alkyl linker attached to the quaternary nitrogen, the linker comprising a carboxy, carbamyl, ureyl, thioureyl or guanidyl group; and
- a bioactive agent which is recognized by a metabolic system attached to the linker.

Another embodiment of the invention is a compound of the formula



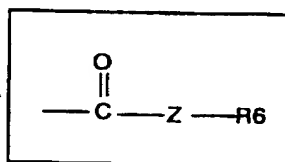
in which

R<sub>1</sub> and R<sub>2</sub> are independently H, linear or

branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, acyl, alkylene, heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, in which the substituent groups are -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms, and k is 0-4;

R<sub>3</sub> and R<sub>4</sub> are independently linear or branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, in which the substituent groups are -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms, and k is 0-4;

R<sub>5</sub> has the structure



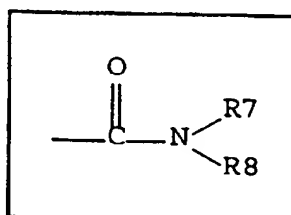
wherein Z is selected from the group consisting of O, S, N, Se, C; and R<sub>6</sub> is absent, or is selected from the group defined for R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub>;

Y is a pharmaceutically acceptable anion;

n is 1 to 6; and

m is 2-10.

Preferably, in the structure defined above, Z is N, and R<sub>5</sub> has the structure



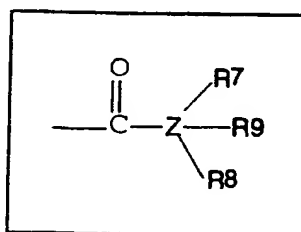
wherein R7 and R8 are independently selected from the group defined for R1, R2, R3 and R4 and optionally further comprise a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono-, di- or polysaccharide, or other bioactive or pharmaceutical agent;

n is 1 to 6;

5 m is 1 to 10; and

Y is a pharmaceutically acceptable anion.

Advantageously, Z is C, and R5 has the structure



15 wherein R7, R8 and R9 are independently selected from the group defined for R1, R2, R3 and R4 and optionally further comprise a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono-, di- or polysaccharide, or other bioactive or pharmaceutical agent;

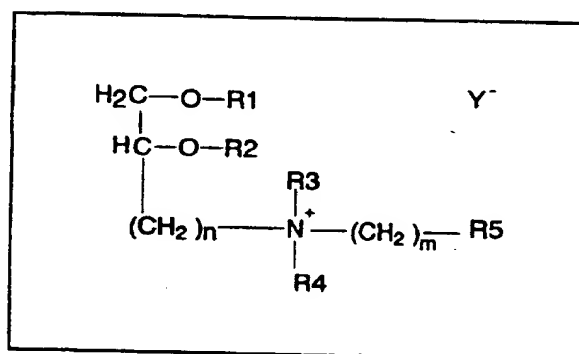
n is 1 to 6;

m is 2 to 10; and

Y is a pharmaceutically acceptable anion.

20 According to another aspect of this preferred embodiment, R1 and R2 are C<sub>8</sub> to C<sub>20</sub> alkyl or alkenyl groups, Z is O and R6 is a chemically linked amino acid or peptide.

The present invention also provides a compound of the formula



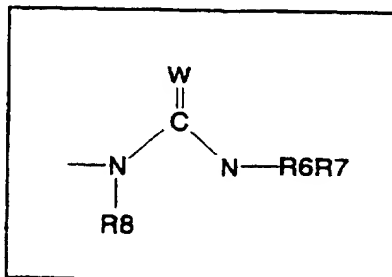
wherein

35 R1, and R2 are independently H, linear or branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, acyl, alkylene, or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, or wherein R1 or R2 may additionally independently be H, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>, wherein

X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

R3 and R4 are independently linear or branched, unsubstituted or substituted  $C_{1-20}$  alkyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, or wherein R1 or R2 may additionally independently be H, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

wherein R5 is  $N-C(O)-$  or R5 has the structure



wherein R6, or R6 together with R7, is selected

from the group defined for R1, R2, R3 and R4 and optionally further comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent;

R8 is absent, or is H or an alkyl group selected from

the group consisting of R1, R2, R3 and R4 and wherein R8 may be joined to R6 or R7 so as to form a ring;

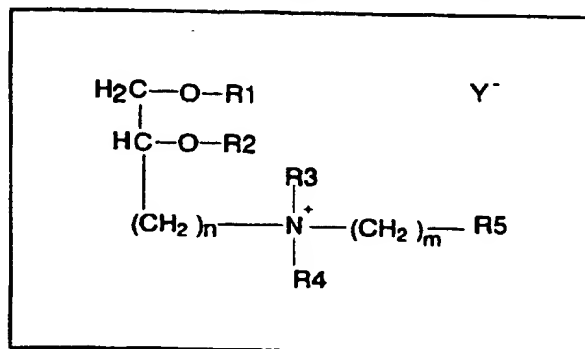
W is O, N, NH, S, Se, C, CH, or  $CR_1R_2$ , wherein  $R_1$  and  $R_2$  are as defined above;

n is 1 to 6;

m is 2 to 10; and

Y is a pharmaceutically acceptable anion.

Another embodiment of the invention is a compound of the formula

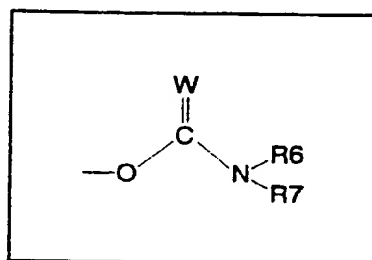




wherein R1 and R2 are independently H, linear or branched, unsubstituted or substituted  $C_{1-23}$  alkyl, acyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

R3 and R4 are independently linear or branched, unsubstituted or substituted  $C_{1-23}$  alkyl or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

wherein R5 has the structure



wherein R6 or R6 together with R7 is selected from the group defined for R1, R2, R3 and R4 and optionally further comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent;

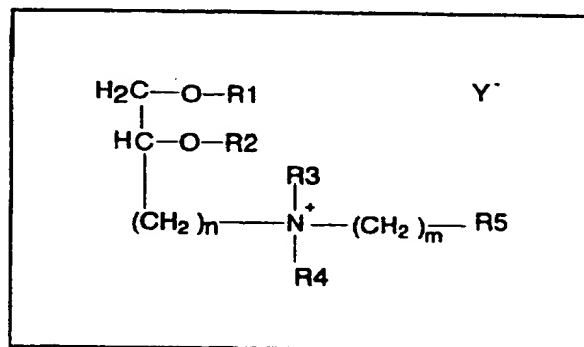
W is O, N, NH, S, Se, C, CH, or  $CR_1R_2$ ;

n is 1 to 6;

m is 2 to 10; and

Y is a pharmaceutically acceptable anion.

Still another embodiment of the invention is a compound of the formula

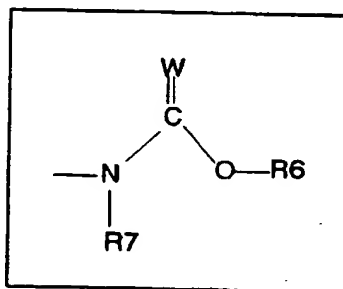


wherein R1 and R2 are independently H, linear or

branched, unsubstituted or substituted  $C_{1-23}$  alkyl, acyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

R3 and R4 are independently linear or branched, unsubstituted or substituted  $C_{1-23}$  alkyl or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

wherein R5 has the structure



wherein R6, or R6 together with R7, is selected from the group defined for R1, R2, R3 and R4 and optionally further comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent;

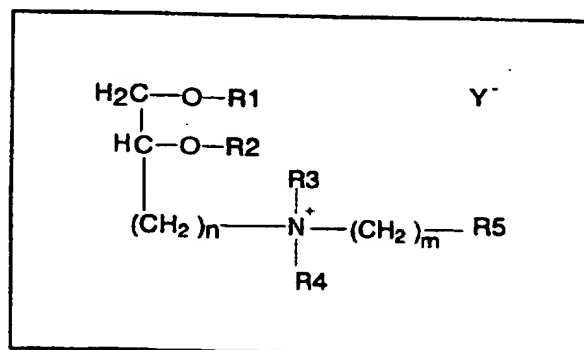
W is O, N, NH, S, Se, C, CH, or  $CR_2$ ;

n is 1 to 6;

m is 2 to 10; and

Y is a pharmaceutically acceptable anion.

The present invention also provides a compound of the formula:



wherein R1 and R2 are independently H, linear or

branched, unsubstituted or substituted  $C_{1-23}$  alkyl, acyl, alkylene, or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

5 R3 and R4 are independently linear or branched, unsubstituted or substituted  $C_{1-23}$  alkyl or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

10 wherein R5 is absent or is  $-O-R6-R7$  or  $-NH-R6-R7$ , wherein R6, or R6 together with R7, is selected from the group defined for R1, R2, R3 and R4 and optionally further comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent;

n is 1 to 6;

m is 2 to 10; and

15 Y is a pharmaceutically acceptable anion.

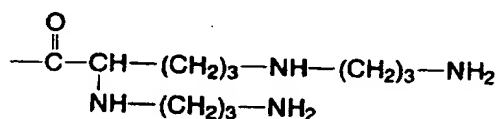
20 Preferably, this compound is selected from the group consisting of amino acids or peptides chemically linked to dialkyl-Rosenthal inhibitor esters and dialkenyl-Rosenthal inhibitor ethers; most preferably, the compound is selected from the group consisting of dialkyl- and dialkenyl-Rosenthal inhibitor ethers covalently linked to a bioactive moiety through a bifunctional linker. Advantageously, R5 is  $-O-R6-R7$ , and R6 is an amino acid or polypeptide. In another preferred embodiment, R5 is glycine.

25 The present invention does not include those compounds wherein R1 is H, or  $C_1-C_{24}$  alkyl or alkenyl and R2 is  $C_1$  to  $C_{24}$  alkyl or alkenyl and R3 and R4 are the same or different and are  $C_1-C_{24}$  alkyl or H, R5 is  $-O-R6-R7$ , and R6 is absent,  $-C(O)-(CH_2)_p-NH$ , a diaminocarboxylate ester group which is alkyl, aryl, or aralkyl or  $-C(O)-(CH_2)_p-NH$  linked to a diaminocarboxylate ester group and p is 1 to 18, and R7 is H, spermine, spermidine, a histone, a protein with DNA-binding specificity or the preceding groups wherein the amine functionalities of the R7 moiety are quaternized with H or a  $C_1$  to  $C_{24}$  straight or branched alkyl chain; or

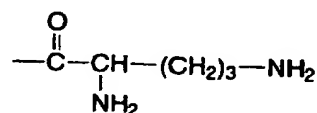
an L- or D-alpha amino acid having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or analogues thereof; or wherein the amine of the R7 moiety is quaternized with H or a  $C_1$  to  $C_{24}$  straight or branched alkyl chain; or

30 a polypeptide selected from the group consisting of L- or D-alpha amino acids, wherein at least one of the amino acid residues comprises arginine, histidine, lysine, ornithine, or analogues thereof.

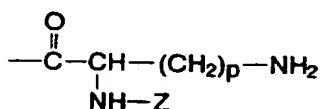
35 Additional compounds not included in the present invention are those wherein n is 1 and m is 2 to 6 and R1 and R2 separately or together are  $C_1-C_{23}$  alkyl or  $C(O)-C_1-C_{23}$ , and R3 and R4 separately or together are H or unbranched alkyl  $C_1-C_6$ , and R5 is  $NH-R6-R7$  and R6-R7 is  $-(CH_2)_zNH_2$  where z is 2-6; or  $-(CH_2)_3-NH-(CH_2)_4-NH_2$ ; or  $NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_5-NH_2$ , C(O)-fluorescein, or



or

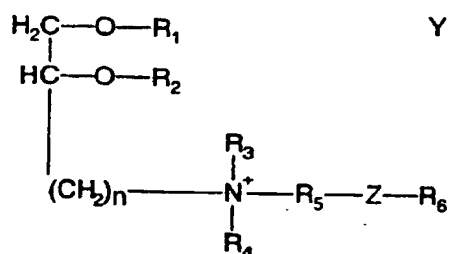


or



where p is 2-5, Z is H or other groups attached by amide or alkyl amino groups.

Another embodiment of the invention is a compound having the structure



wherein R1 and R2 are independently H, linear or branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, acyl, alkylene, or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

R3 and R4 are independently linear or branched, unsubstituted or substituted  $C_{1-23}$  alkyl or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, the groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of the substituents comprise from 0 to 2 heteroatoms and k is 0-4;

5 R5 is absent or is  $-O-R_6-R_7$  or  $-NH-R_6-R_7$ ,  
wherein R6, or R6 together with R7, is selected from the group defined for R1, R2, R3 and R4 and optionally further comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent; and

n is 1 to 6;

10 m is 2 to 10; and

Y is a pharmaceutically acceptable anion; and

Z is O, NH, or S;

Preferably, R5 is a heteroethylene group, X is O and R6 together with R7 is a amino acid or peptide linked to the heteroethylene group through an ester bond.

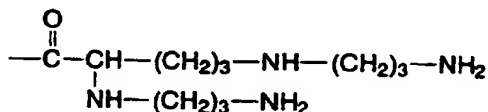
15 The present invention does not include certain compounds which would fall within the structure above. For example, if Z is O and R1 is H, or  $C_1$ - $C_{24}$  alkyl or alkenyl and R2 is  $C_1$  to  $C_{24}$  alkyl or alkenyl and R3 and R4 are the same or different and are  $C_1$ - $C_{24}$  alkyl or H, and R6 is absent,  $-C(O)-(CH_2)_p-NH$ , a diaminocarboxylate ester group which is alkyl, aryl, or aralkyl or  $-C(O)-(CH_2)_p-NH$  linked to a diaminocarboxylate ester group and p is 1 to 18, and R7 is H, spermine, spermidine, a histone, a protein with DNA-binding specificity or the preceding groups wherein the amine functionalities of the R7 moiety are quaternized with H or a  $C_1$  to  $C_{24}$  straight or branched alkyl chain; or

20 an L- or D- $\alpha$  amino acid having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or analogues thereof; or

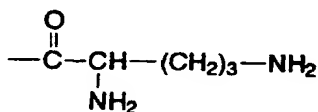
wherein the amine of the R7 moiety is quaternized with H or a  $C_1$  to  $C_{24}$  straight or branched alkyl chain; or

25 a polypeptide selected from the group consisting of L- or D- $\alpha$  amino acids, wherein at least one of the amino acid residues comprises arginine, histidine, lysine, ornithine, or analogues thereof.

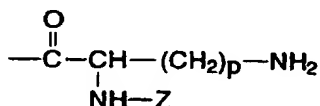
Additional compounds not included in the present invention are those wherein Z is NH and n is 1 and m is 2 to 6 and R1 and R2 separately or together are  $C_1$ - $C_{23}$  alkyl or  $C(O)-C_1-C_{23}$ , and R3 and R4 separately or together are H or unbranched alkyl  $C_1$ - $C_6$ , and R5 is  $NH-R_6-R_7$  and R6-R7 is  $-(CH_2)_zNH_2$  where z is 2-6; or  $-(CH_2)_3NH-(CH_2)_4NH_2$ ; or  $-NH-(CH_2)_3NH-(CH_2)_4NH(CH_2)_3NH_2$ , C(O)-fluorescein, or



35 or

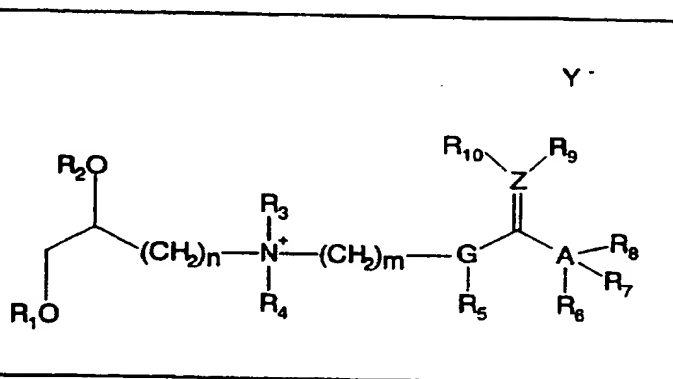


or



where p is 2-5, Z is H or other groups attached by amide or alkyl amino groups.

The present invention also provides a compound of the formula:



(Formula 1)

wherein

$\text{R}_1$  and  $\text{R}_2$  are independently linear, branched, unsubstituted or substituted  $\text{C}_{1-23}$  alkyl, acyl, alkylene, or heteroalkyl groups having from 0 to 6 sites of unsaturation, or cyclic or aryl groups, said cyclic or aryl groups containing up to five heteroatoms, wherein the substituent groups are selected from the group consisting of  $-\text{O}(\text{CH}_2)_k-$ ,  $\text{CH}_3$ ,  $-\text{S}(\text{CH}_2)_k-\text{CH}_3$ ,  $\text{X}(\text{CH}_2)_k-$ , wherein X is a halide, and  $-\text{N}((\text{CH}_2)_k-\text{CH}_3)_2$ , wherein k is 0 to 4, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms; and  $\text{R}_1$  or  $\text{R}_2$  may be H;  $\text{R}_3$  and  $\text{R}_4$  are defined in the same manner as  $\text{R}_1$  and  $\text{R}_2$  with the exception that  $\text{R}_3$  or  $\text{R}_4$  may not be H;  $\text{R}_5$  is absent, H or an alkyl group as defined for  $\text{R}_1$  and  $\text{R}_2$ ;  $\text{R}_5$  through  $\text{R}_{10}$  independently or in combination are absent, or are H or alkyl groups as defined for  $\text{R}_1$  and  $\text{R}_2$  and, optionally, further comprise a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono-, di- or polysaccharide, or other bioactive or pharmaceutical agent; G is absent, O, N, S or Se; Z is O, N, S, Se, C; A is O, N, S, Se, C; n is 1 to 6; m is 2-10, and Y is a pharmaceutically

acceptable anion. The present invention does not include the compounds of Formula 1 wherein G is N, Z is O and A is C or those compounds of Formula 1 wherein G is O, Z is O and A is C.

The present invention also provides cytofectin formulations comprising any of the cationic lipids of described above and a co-lipid selected from the group consisting of neutral lipids, phospholipids, and cholesterol in a suitable carrier solution.

Still another embodiment of the invention is a method of delivering an anionic molecule into a cell comprising the steps of:

(a) contacting the anionic molecule with a formulation comprising an effective amount of any of the cationic lipids described above to form a complex with the lipid; and

(b) contacting cells with the lipid complex formed in step (a);

whereby a biologically effective amount of the anionic molecules are inserted into the cells. Preferably, said cells are *in vitro*; most preferably, said cells are *in vivo*.

Another aspect of the present invention is the use of the compound of Formula 1 above in a composition for introducing a bioactive agent into a cell. Preferably, the cell is *in vivo*. In one embodiment, the cell is *in vitro*.

In one embodiment of the use, the bioactive agent is anionic, and may be DNA or RNA.

Activity of the various compounds may be demonstrated, for example, in an assay such as an *in vitro* cell culture assay, murine lung transfection, murine intraperitoneal tumor, and murine intramuscular and porcine or rabbit intraarterial assays. Such assays are illustrated herein as screening techniques for the claimed compounds.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a graph of the level of total  $\beta$ -gal expression obtained in COS7 cells with different ratios of GMU-DMRIE:DOPE.

Figure 1B is a graph of the peak  $\beta$ -gal expression obtained in COS7 cells with different ratios of GMU-DMRIE:DOPE.

Figure 1C is a graph of the total  $\beta$ -gal expression obtained in C2C12 cells with different ratios of GMU-DMRIE:DOPE.

Figure 1D is a graph of the peak  $\beta$ -gal expression obtained in C2C12 cells with different ratios of GMU-DMRIE:DOPE.

Figure 2 is a graph of pulmonary expression of CAT in mouse lung when transfection was mediated with cytofectins having alkylamine moieties of differing chain length.

Figure 3 is a graph of pulmonary expression of CAT in mouse lung when transfection was mediated with cytofectins having alkyl chains of C<sub>10-14</sub>.

Figure 4 shows the activities of the amino acid linked compounds Gly-G-DLRIE and Lys-G-DLRIE relative to GAP-DLRIE in the murine lung assay.

Figure 5 is a graph comparing intraperitoneal transfection activity of three cytofectins in a mouse tumor model.

### BRIEF DESCRIPTION OF REACTION SCHEMES

Following the text of the specification are several representative reaction schemes. These numbered reaction schemes illustrate the following:

Scheme I illustrates the synthetic pathway for the basic skeleton of the cytofectins of the present invention.

Scheme II illustrates routes for the preparation of intermediates from commercially available starting materials in the synthesis of the cytofectins of the invention.

Scheme III illustrates the synthetic pathway for carboxy cytofectins from an intermediate compound.

Scheme IV illustrates the synthetic pathway for carbamyl cytofectins from an intermediate compound.

Scheme V illustrates the synthetic pathway for cytofectins with urea-like functional groups from an intermediate compound.

### DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that amphipathic lipids of the quaternary ammonium class of cytofectins, for which the Rosenthal Inhibitor (RI) series is the paradigm, can be derivatized to form highly effective transfection agents having the ability to interact more specifically with the cell membrane and to achieve higher levels of transfection. They provide structures that can be adapted to target key receptors and enzymes of cellular surfaces and are thus suitable for use in the discovery and exploitation of important factors in molecular recognition. Some of these cationic lipids can also be attached to substances that are delivered intracellularly for achieving a particular biological purpose.

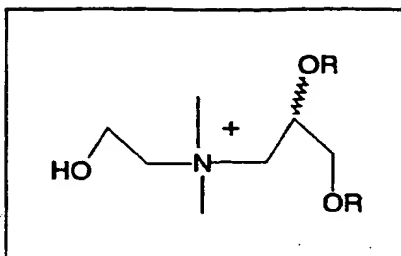
The cationic lipids of the present invention have chemical structures comprising the DOTMA or RI features as described previously, but have attached thereto, through a linking spacer, a structure advantageously having functional groups, for example, a ketone, ester, ureyl or carbamyl moiety. The functional group can be used either (1) to attach a cell targeting moiety or (2) to attach a therapeutic molecule to the cytofectin. Additionally or alternatively, the functional group can be used as a linker to attach groups that can increase the polar charge density of the cytofectin, thus enhancing transfection. For example, we have discovered that the presence of a primary amine group within 8 carbons of the quaternary nitrogen has been found to enhance transfection efficiency. Examples of effective cytofectins having a primary amine within 8 carbons of the quaternary nitrogen are disclosed in Examples 7 and 9, and the results shown in Figures 2, 3, 4, and 5.

Despite the original presumption that only positively charged amino acids or peptides would be capable of increasing cytofectin efficiency (see U.S. patent 5,264,618), transfection efficiency may also be enhanced by linking a peptide or amino acid which is non-polar, polar and uncharged, or negatively charged at physiological pH to the Rosenthal Inhibitor backbone. For example, the uncharged amino acid glycine confers greater transfection activity when linked to DLRIE than does the charged amino acid lysine.

#### Nomenclature

The cytofectins of the invention are cationic lipid species which have the core structure of the RI, DL-2,3-diacyloxypropyl(dimethyl)- $\beta$ -hydroxyethylammonium





Examples of acronyms for the RI class of cytofectins are:

**DORI:** DiOleylRosenthalInhibitor  $R = \text{CO}(\text{CH}_2)_{7[2]}\text{CH}-(\text{CH}_2)_7\text{CH}_3$

10 **DORIE:** DiOleylRosenthalInhibitorEther  $R = (\text{CH}_2)_9[2]\text{CH}-\text{CH}(\text{CH}_2)_7\text{CH}_3$

**DDRIE:** DiDodecylRosenthalInhibitorEther  $R = (\text{CH}_2)_9\text{CH}_3$

**DLRIE:** DiLaurylRosenthalInhibitorEther  $R = (\text{CH}_2)_{11}\text{CH}_3$

**DMRIE:** DiMyristylRosenthalInhibitorEther  $R = (\text{CH}_2)_{13}\text{CH}_3$  **DPRIE:** DiPalmitylRosenthalInhibitorEther  
 $R = (\text{CH}_2)_{15}\text{CH}_3$

15 **DSRIE:** DiStearylRosenthalInhibitorEther  $R = (\text{CH}_2)_{17}\text{CH}_3$

**ARE-DMRIE:**  $\beta$ -AminoEthylDiMyristylRosenthalInhibitorEther

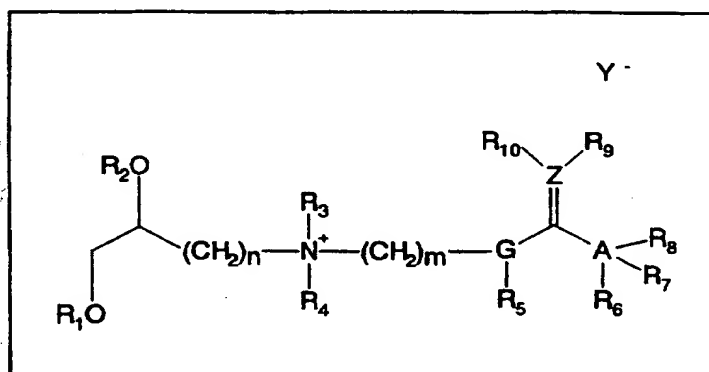
**DMRIE-Ox:** DiMyristylRosenthalInhibitorEtherCarboxylate

Similar acronyms denote neutral lipids contained in liposomal formulations, for example:

**DOPE:** DiOleoylPhosphatidylEthanolamine

20 **Cytofectins of the Invention: Structure**

In one embodiment, the lipids of the invention have the general structure:



(Formula 1)

wherein

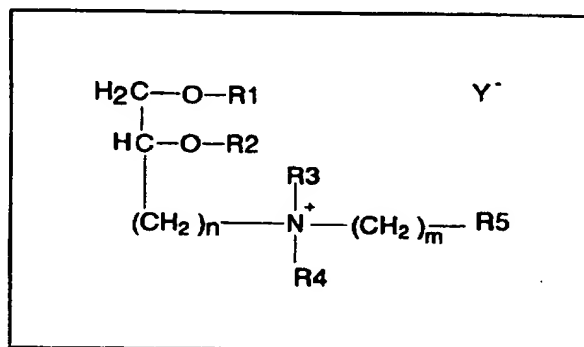
$R_1$  and  $R_2$  are independently linear, branched, unsubstituted or substituted  $C_{1-23}$  alkyl, acyl, alkylene, or heteroalkyl groups having from 0 to 6 sites of unsaturation, or cyclic or aryl groups, said cyclic or aryl groups containing up to five heteroatoms, wherein the substituent groups are selected from the group consisting of  $-\text{O}-(\text{CH}_2)_k-$ ,  $\text{CH}_3$ ,  $-\text{S}-(\text{CH}_2)_k-\text{CH}_3$ ,  $\text{X}-(\text{CH}_2)_k-$ , wherein X is a halide, and  $-\text{N}((\text{CH}_2)_k-\text{CH}_3)_2$ , wherein the alkyl groups of said substituents

comprise from 0 to 2 heteroatoms and k is 0 to 4; and R<sub>1</sub> or R<sub>2</sub> may be H; R<sub>3</sub> and R<sub>4</sub> are defined in the same manner as R<sub>1</sub> and R<sub>2</sub> with the exception that R<sub>3</sub> or R<sub>4</sub> may not be H; R<sub>5</sub> is absent, H or an alkyl group as defined for R<sub>1</sub> and R<sub>2</sub>; R<sub>5</sub> through R<sub>10</sub> independently or in combination are absent, or are H or alkyl groups as defined for R<sub>1</sub> and R<sub>2</sub> and, optionally, further comprise an amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono-, di- or polysaccharide, or other bioactive or pharmaceutical agent chemically linked thereto; G is absent, O, N, S or Se; Z is O, N, S, Se, C; A is O, N, S, Se, C; n is 1 to 6; m is 2-10, and Y is a pharmaceutically acceptable anion.

The present invention does not include the compounds of Formula 1 wherein G is N, Z is O and A is C or those compounds of Formula 1 wherein G is O, Z is O and A is C or those in which G, Z, and A are C.

In a preferred embodiment of this structure, n=1-2. In another preferred embodiment, m=2-4. In a further preferred embodiment, k=0-4. Preferentially, R<sub>3</sub>-10, if alkyl, are C<sub>10</sub>-15.

In a preferred embodiment, the cytofectins of the present invention have the following formula:



(Formula 2)

wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub> are independently linear or branched, unsubstituted or substituted C<sub>1-20</sub> alkyl, acyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, and containing from 0 to 5 heteroatoms, the substituent groups selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>), wherein the alkyl groups contain from 0 to 2 heteroatoms; n is 1 to 6; m is 2 to 10; and R<sub>5</sub> is a chemical structure having functional groups that define a species of formula 2. R<sub>5</sub> is preferably linked to the ammonium nitrogen through an alkyl linker, which can also contain heteroatoms.

The cationic lipids of the invention are associated with a physiologically acceptable non-toxic anion, Y. Anions commonly used in pharmaceutical preparations are disclosed in Berge et al. (*J. Pharm. Sci.* 66:1-19, 1977), which is hereby incorporated by reference.

In preferred embodiments, n is 1 and m is 2-4, and the preferred cytofectin species of the invention thus have the RI skeleton shown in the key intermediate, formula 1-3, *infra*. Other cytofectin species of the present invention are homologs of the RI based group, and these cytofectins have the same general formula 1; however, n is 3-6 and m is 2-10.

The cytofectins of the present invention can be viewed as an assembly of functional parts in a modular arrangement, comprising:

A. A hydrophobic structure, which can be aliphatic chains, that can include cyclic structures within, but not between, the aliphatic chains.

5 B. A most characteristic portion of the molecule, comprising a quaternary N atom substituted with dialkoxy, diacyloxy, and alkyl groups, that can also be part of a ring that includes the groups attached above and below in Formula 1.

C. A spacer,  $(CH_2)_n$ , that is usually an aliphatic chain, but can also include one or more heteroatoms.

10 D. A linker group, often a backbone of three atoms having a central carbon atom, usually doubled bonded to oxygen. The linker group is carboxyl, carbamyl, ureyl, or guanidyl, and, as will be considered below, may have a common key synthetic intermediate. In the molecules of the invention, when this group is not symmetrical, its left to right orientation can be reversed.

15 E. A "cargo" portion of the molecule, which can comprise amino acids, peptides, proteins, carbohydrates, nucleic acids, drugs, ligands, or any other molecular species that can interact with a cell constituent to induce a desired response. These molecules can be attached to the linker group of the molecule by bifunctional spacers.

20 Compounds of the class described above were designed to allow efficient derivatization of the basic dioxopropaniminium skeleton with a wide variety of chemical moieties, and in particular those entities which are considered physiologically active. Members of this class of cytofectins have been constructed as paradigms for the general introduction of a wide variety of chemical functionalities using the synthetic methods disclosed herein. The unique functional groups described herein as "linkers" will also impart enhanced properties of efficacy and/or specificity to the cytofectins of the invention.

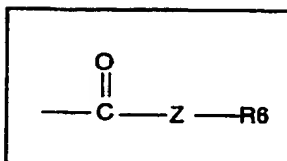
25 The specific bioactive peptides and saccharides introduced into the ureas and thioureas, respectively, will allow selective targeting or enhanced internalization via receptor-mediated processes. The introduction of a urea group will increase the interaction between the cytofectin and the phosphate backbone of DNA, thus enhancing transfection. The ability to generalize the disclosed procedures will allow easy functionalization of the basic cytofectin skeleton with a wide variety of bioactive molecules known to one of ordinary skill in the art.

Species of the cytofectins of the present invention are classified on the basis of a characteristic R5 substitution in the general formula 1, as follows:

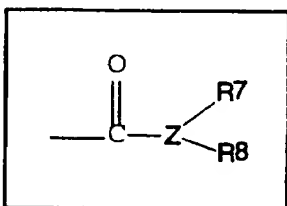
30 1. Carboxy cytofectins

One species of cationic lipids of the invention is characterized by the presence of a carboxy group in a substituent linker group on the ammonium group of formula 1. The members of this class have the general structure of formula 1, however, in the carboxy species R5 has the structure

wherein Z is O, N, S, Se, C or H; and R6 is the group defined for R1, R2, R3 or R4;

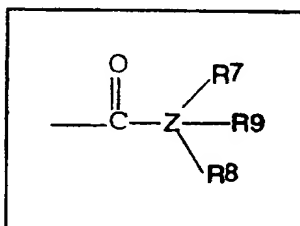


5 When Z is N, R5 has the structure



10

and when Z is C, R5 has the structure



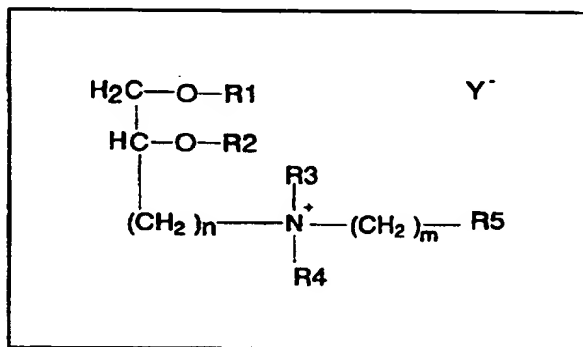
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wherein R7, R8 and R9 are independently H or are selected from the group defined for R1, R2, R3 and R4.

20 In all members of this species R6, R7, R8 and R9 optionally further comprise a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono-, di- or polysaccharide, or other bioactive or pharmaceutical agent.

## 2. Carbamyl cytofectins

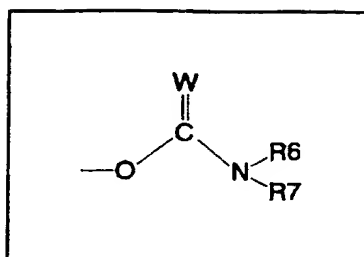
25 Another species of cationic lipids of the invention is characterized by the presence of a carbamyl group in one of the substituents on the ammonium group of formula 1 and the members of this class have the general structure



30

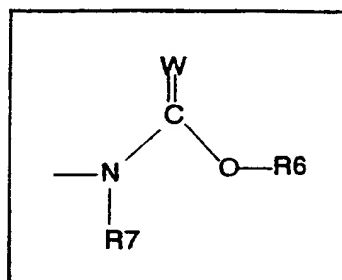
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wherein R5 has the structure



wherein W is O, N, NH, S, Se, C, CH, CR<sub>1</sub> or CR<sub>1</sub>R<sub>2</sub> and R<sub>6</sub> and R<sub>7</sub> are as defined above.

The carbonyl cationic lipids of the invention also include those having the isomeric carbamyl structure wherein R<sub>5</sub> has the structure

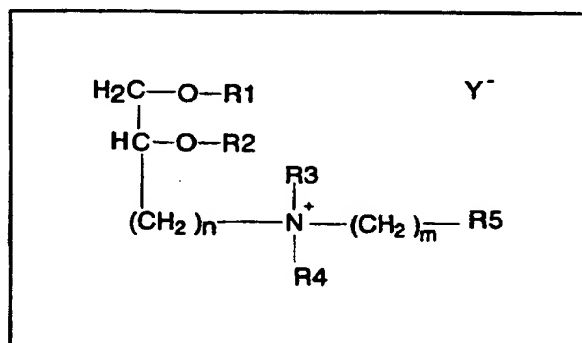


wherein W is as defined above, R<sub>6</sub> is as defined for the carboxy species and R<sub>7</sub> is absent, or is H or an alkyl group as defined for the carboxy species. Preferred embodiments of the carbamate cytofectins comprise methyl carbamate groups attached to the lipid through alkyl linkers (CH<sub>2</sub>)<sub>m</sub> wherein m is 2 to 4.

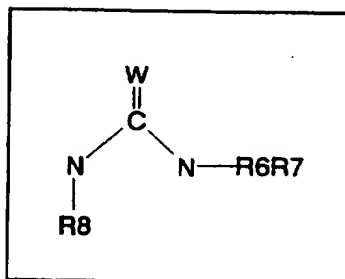
The carbamate structure allows facile amine-alcohol ligand coupling at the terminal group.

### 3. Cytofectins having urea-like linking groups

Another species of cationic lipids of the invention is characterized by the presence of a urea-like group in a substituent on the ammonium group of formula 1 and the members of this class have the general structure:



wherein R<sub>5</sub> has the structure



5 wherein W is O, N, NH, S, Se, C, CR1, CR1R2; R6 and R7 are independently as defined for the carboxy species

above; R8 is absent, or is H or an alkyl group R1, R2, R3, or R4 as defined above for formula 1; and when R6, R7 and R8 are not H, they optionally further comprise a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono-, di- or polysaccharide, or other bioactive or pharmaceutical agent.

(a) Ureyl cytofectins

One species of the cationic lipids of the invention of this class thus has the general structure of formula 1 and is characterized by the presence of a ureyl group in the substituent on the ammonium nitrogen of formula 1. In this species R5 has the structure as defined above wherein W is oxygen.

(b) Guanidyl cytofectins

Another species of cationic lipids of the invention according to this class are characterized by the presence of a guanidyl group in a substituent of the ammonium group nitrogen of formula 1 and have the general structure of formula 1 wherein R5 has the structure as defined above wherein W is N or NH.

(c) Thiourea and selenourea cytofectins

The cationic lipids of the invention also include compounds having the general structure of formula 1 wherein R5 has the structure as defined above wherein W is S or Se.

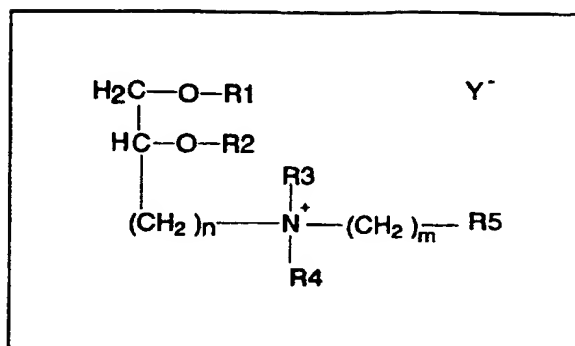
(d) Other urea-related cytofectins

The cationic lipids of the invention also include compounds having the general structure of formula 1 wherein R5 is as defined above wherein W is C, CH, CR1, or CR1R2, wherein R1 and R2 are as defined for formula 1; R6, R7 and R8 are selected from the group defined for R1, R2, R3 and R4

The urea/thiourea synthesis allows facile amine-amine ligand coupling, and provides a means to prepare saccharide and amino acid derivatives. Preferred saccharide derivatives that can be prepared via the thiourea linkage include glucose, galactose, lactose, and arabinose species.

4. N<sup>+</sup> heteroethylene substituted cytofectins

The invention also includes another major species of cationic lipids comprising a heteroethylene substitution on the quaternary ammonium of a propaminium group including the  $\beta$ -hydroxyethylene substitution that is characteristic of the RI species of cationic lipid, as well as comprising the amine derivative disclosed in Wheeler et al., Biochem. Biophys. Acta, in press. This group of compounds has the general structure



wherein R1 and R2 are independently H, linear or branched,

unsubstituted or substituted C<sub>1,23</sub> alkyl, acyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms k is 0-4, n is 1-6, and m is 2-10.

R3 and R4 are independently linear or branched, unsubstituted or substituted C<sub>1,23</sub> alkyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0-4;

wherein R5 is H or is -O-R6-R7 or -NH-R6-R7, wherein R6, or R6 together with R7, is selected from the group defined for R1, R2, R3 and R4 and optionally further comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent;

and Y is a pharmaceutically acceptable anion;

The present invention does not encompass compounds known in the art which fall within the generic structure above. For example, the present invention does not include those compounds wherein R5 is -O-R6-R7 and R1 is H, or C<sub>1</sub>-C<sub>24</sub> alkyl or alkenyl and R2 is C<sub>1</sub> to C<sub>24</sub> alkyl or alkenyl and R3 and R4 are the same or different and are C<sub>1</sub>-C<sub>24</sub> alkyl or H, and R6 is absent, -C(O)-(CH<sub>2</sub>)<sub>p</sub>-NH, a diaminocarboxylate ester group which is alkyl, aryl, or aralkyl or -C(O)-(CH<sub>2</sub>)<sub>p</sub>-NH linked to a diaminocarboxylate ester group and p is 1 to 18, and R7 is H, spermine, spermidine, a histone, a protein with DNA-binding specificity or the preceding groups wherein the amine functionalities of the R7 moiety are quaternized with H or a C<sub>1</sub> to C<sub>24</sub> straight or branched alkyl chain; or

an L- or D-α amino acid having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or analogues thereof; or

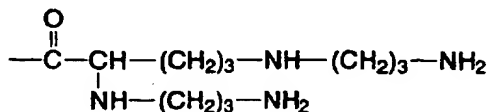
wherein the amine of the R7 moiety is quaternized with H or a C<sub>1</sub> to C<sub>24</sub> straight or branched alkyl chain; or

a polypeptide selected from the group consisting of L- or D-α amino acids, wherein at least one of the amino acid residues comprises arginine, histidine, lysine, ornithine, or analogues thereof.

Additional compounds not included within the present invention are those wherein R5 is NH-R6-R7 and n is 1 and m is 2 to 6 and R1 and R2 separately or together are C<sub>1</sub>-C<sub>23</sub> alkyl or C(O)-C<sub>1</sub>-C<sub>23</sub>, and R3 and R4 separately

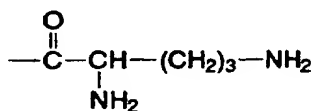
or together are H or unbranched alkyl C<sub>1</sub>-C<sub>6</sub>, and R5 is NH-R6-R7 and R6-R7 is -(CH<sub>2</sub>)<sub>z</sub>NH<sub>2</sub> where z is 2-6; or -(CH<sub>2</sub>)<sub>3</sub>-NH-(CH<sub>2</sub>)<sub>4</sub>-NH<sub>2</sub>; or -NH-(CH<sub>2</sub>)<sub>3</sub>-NH-(CH<sub>2</sub>)<sub>4</sub>-NH(CH<sub>2</sub>)<sub>3</sub>-NH<sub>2</sub>, C(O)-fluorescein, or

5



or

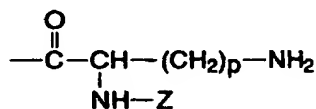
10



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or

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where p is 2-5, Z is H or other groups attached by amide or alkyl amino groups.

In preferred embodiments, the RI lipid moiety is linked to amino acids or polypeptides through an alkyl linker.

25 Alternatively, the cytofectin can include a bifunctional linker, for example hydroxysuccinyl-DORIE-propyl amide.

#### Bioactive Headgroups on the Cytofectins

##### (a) Targeting species

A cytofectin according to the present invention can include a molecular species having a biological cell targeting activity as a terminal group. Within this class are cytofectins comprising cell receptor-specific molecules.

30 The receptor-specific peptides or amino acids are typically linked as amides via DMRIE-OX and γAP-DMRIE. Examples of preferred species of this type are DMRIE carboxylate (methionine-leucine-phenylalanine methyl ester) amide (DOx-Met-Leu-Phe-OMe), and pGlu-Pro-His-γ-DMRIE, comprising chemotactic peptides. Other ligands for cell surface receptors that can be attached to cytofectins of the invention comprise peptidomimetic analogs; many viral attachment and internalization peptides, lactose and other di- and polysaccharides; acetylcholine analogs; and folic acid derivatives.

##### (b) Therapeutic agents



A cytofectin according to the invention can include as a terminal group a bioactive molecular species. An example of a preferred species of this type is pGlutamate-histidine-proline- $\gamma$ -DMRIE amide, comprising a thyrotropin-releasing hormone.

(c) Cellular and Intracellular Targeting

5 A cytofectin according to the invention can comprise a terminal group bearing a ligand that can specifically bind to a cell membrane or intracellular target to effect a desired physiological response. Appropriate ligands may comprise peptides that are viral epitopes, hormones, enzyme substrates, monosaccharides, disaccharides, oligosaccharides, carbohydrates, cofactors, drugs, lectins, oligonucleotides, and nucleic acids. Preferred species among this group are cytofectins comprising chloroquine and other lysosomotropic agents, nuclear localization  
10 peptides, corticosteroids and viral peptides or proteins.

Groups influencing transfection efficiency

The cytofectins of the present invention may be linked to groups which influence their transfection efficiencies. Such groups may be amino acids, peptides, polypeptides, proteins, nucleic acids, nucleotides, polynucleotides, mono, di- or polysaccharides. Additionally, the amino acids, peptides, polypeptides, or proteins may  
15 include unusual or modified amino acids not generally found in living organisms. Such unusual or modified amino acids include but are not limited to the modified and unusual amino acids listed in 37 C.F.R. § 1.822. In addition, such amino acids may be synthetic amino acids not found in nature.

Transfection Efficacy

20 Representative compounds of each class in the DMRIE series together with a measurement of cytofectin activity relative to DMRIE are listed in Table I. Table I clearly demonstrates that DMRIE derivatives having several of the linking groups of the present invention provide efficient levels of transfection.

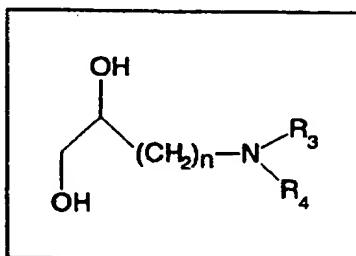
## I: SUMMARY OF CYTOFECTIN HEADGROUP ALTERATIONS AND ACTIVITY

	Functional Group	Alkyl Link	Name	Activity	
				Cos7	C2C12
5	Methyl Ureas	ethyl (C2)	BMU	≈	≈
		propyl (C3)	GMU	≈	≈
		butyl (C4)	DMU	≈	≈
	Methyl Carbamates	ethyl (C2)	βMC	≈	≈
		propyl (C3)	γMC	≈	≈
		butyl (C4)	δMC	≈	≈
10	Sugar-thiourea				
		glucose	Glc-TU-DMRIE	≈	≈
		arabinose	Ara-TU-DMRIE	≈	<
		galactose	Gal-TU-DMRIE	≈	≈
	Single Amino Acids				
		serine	δ-ser-DMRIE	≈	≈
15	Peptides	methionine	DOX-Met-OMe	≈	<
		Met-Leu-Phe	DOX-MLF-OMe	≈	<
		pGlu-His-Pro	TRH-γ-DMRIE	≈	≈

20 Reaction Schemes and Preparation MethodsA. Synthesis of Cytofectins

The compounds of the invention may be prepared by any convenient process. In order to expediently enable the synthesis of the cytofectins of the invention from the key intermediates of Scheme II, generalized methods for the synthesis of these derivatives are presented diagrammatically and in terms of specific examples. The various carboxy cytofectins, both of the carbamyl cytofectin species, and the cytofectins with urea-like functional groups can be prepared according to the synthesis procedures outlined in Schemes III, IV, and V, respectively.

The cytofectins of the invention are conveniently prepared from homologs of a key intermediate having the general formula



Compound I-1

Preferred cytoflectins wherein n is 1 and m is 2 are prepared from the Compound I-3 of Scheme I, wherein n is 1. Compound I-3 can be prepared from I-1 by alkylation at the hydroxyls of dialkylaminopropanediol to generate I-2 followed by alkylation of N with an R-substituted ethyl group as shown.

Scheme II sets forth a general method of synthesis of the starting compounds for Scheme I for species wherein n=1-6. Various compounds along the route of synthesis are commercially available, for example as the hydroxy-olefin II-3 or the bromo-olefin II-4. Only the cytoflectin species wherein n = 5 requires starting from the acid II-1. For the preparation of the preferred species wherein n is 1 and m is 2, the final dimethylaminopropanediol is commercially available (Cat. No. 21,021-8, Aldrich Chemical Co., St. Louis, MO).

The cytoflectins of the invention are prepared by appending various known molecules with reactive functional groups in an appropriate order to the skeleton structure of the key intermediates. The various known compounds, for example, substituted alkyl amines, sugars, ureas, thioureas, amino acids, and peptides are available from commercial sources. Methods for coupling the various functional groups to the key intermediates are well known to those skilled in the art and described in detail in the literature, for example in the monograph series, Reagents for Organic Synthesis Vol. 1-16, John Wiley & Sons, New York, NY.

#### Summary of Synthetic Transformations

Cytoflectins of the type claimed in this application may be prepared using conventional synthetic chemistry. The synthetic procedures for all of the cytoflectins disclosed herein is essentially the same, but employ different amino-alcohols as starting materials. The skeleton is formed by elaboration of dihydroxy-dialkylamine compounds as illustrated in Scheme I. The hydroxyl functions are linked to hydrophobic alkyl chains using reagents and conditions dictated by the specific substituents desired to be introduced. For example, simple n-alkyl chains may be conveniently introduced by coupling the requisite alkyl methane sulfonate with the alcohols using base catalysis. The tertiary amine is then treated with a functionalized, suitably protected alkylating agent to effect quaternization of the nitrogen. Specific functionalities appended via the quaternary nitrogen may then be elaborated into the various functional classes called Carboxy-, Carbamyl-, and Urea-Cytoflectins, as shown in Schemes III, IV, and V. General synthetic strategies such as those disclosed in U.S. Patent No. 5,334,761, which is incorporated herein by reference, can be used to advantage in the present invention.

To prepare Carboxy Cytoflectins according to Scheme III, a primary alcohol moiety linked to the quaternary nitrogen (III-1) is oxidized to the corresponding carboxylic acid (III-2). Numerous reagents may effect this transformation, and we standardly employ a modified chromium trioxide oxidation. The cytoflectin carboxylate is typical in that it may be coupled with a variety of alcohols, thiols, and amines to afford the corresponding esters, thioesters, and amides, respectively. In this manner, any material bearing an appropriate nucleophile may be linked to the "basic" carboxy cytoflectin skeleton to generate compounds such as III-3 and III-4. For example, the preparation of DMRIE carboxylate propyl amide was effected by DCC-catalyzed coupling of propyl amine with DMRIE carboxylate. The corresponding amino acid and peptide derivatives were prepared using similar technology, and provide further specific illustrations of this type of synthetic route and its application in incorporating biologically active moieties.

Two "orientations" are possible when preparing Carbamyl Cytoflectins, and synthetic routes for both orientations are illustrated in Scheme IV. Alcohol moieties appended to the quaternary nitrogen (IVA-1) may be converted to the corresponding carbamates (IVA-2) in several ways, including treatment with an appropriate isocyanate. Amine substituents appended to the quaternary nitrogen (IVB-1) may also be converted to carbamates (IVB-2) in an analogous manner, although different reagents are typically required. For example, the preparation of the methyl carbamate of DMRIE employed coupling the parent cytoflectin alcohol with methylisocyanate.

The Urea Cytoflectins (V-2) may be prepared from cytoflectins bearing a primary or secondary amine function (V-1) according to Scheme V. This may be done in several ways. For example, we have employed both single step conversions using various isocyanates and two step conversions comprised of initial treatment with phosgene followed by coupling with an amine. The preferred methodology for any given compound depends on the functional groups within the starting cytoflectin and those present on the amine substituents. For example,  $\gamma$ -amino propyl DMRIE may be treated with methylisocyanate to yield the corresponding methyl urea in one step. Alternately, the two step conversion technique was employed to generate the  $\gamma$ -amino propyl DMRIE arginine methyl ester urea.

Following these generalized procedures, the following cytoflectins were among those prepared and tested for transfective activity:

1. RI cytoflectins

Serine- $\delta$ -DMRIE amide  
Aspartate- $\gamma$ -DMRIE amide  
pGlutamate-histidine-proline- $\gamma$ -DMRIE amide  
(a thyrotropin releasing hormone derivative  
Hydroxysuccinyl-DORIE Propyl Amide

2. Carboxy Cytoflectins

DORIE Carboxylate [C18:1 alkenyl side chains]  
DMRIE Carboxylate [C14 alkyl side chains]  
DMRIE Carboxylate Propyl Amide  
DMRIE Carboxylate (methionine-methyl ester) amide  
DMRIE Carboxylate (methionine-leucine-methylester) amide  
DMRIE Carboxylate (methionine-leucine-phenyl alanine methyl ester) amide [a chemotactic peptide derivative]

3. Carbamyl Cytoflectins

DMRIE Methyl carbamate  
Hydroxypropyl DMRIE Methyl Carbamate  
Hydroxybutyl DMRIE Methyl Carbamate

4. Urea-like Cytoflectins

Ureas  
 $\beta$ -aminoethyl-DMRIE, Methyl Urea

$\gamma$ -aminopropyl-DMRIE, Methyl Urea  
 $\delta$ -aminobutyl DMRIE, Methyl Urea  
 $\gamma$ -aminopropyl-DMRIE, Arginine-Methyl Ester  
 $\gamma$ -aminopropyl-DMRIE, Lysine Methyl Ester Urea  
 $\gamma$ -aminopropyl-DMRIE, Lysine Inner Salt Urea

Urea

#### Thioureas

$\gamma$ -aminopropyl-DMRIE,  $\alpha$ -glucosyl thiourea  
 $\gamma$ -aminopropyl-DMRIE,  $\alpha$ -galactosyl thiourea  
 $\gamma$ -aminopropyl-DMRIE,  $\alpha$ -arabonysl thiourea  
 $\gamma$ -aminopropyl-DMRIE,  $\alpha$ -lactosyl thiourea  
 DMRIE- $\gamma$ -thiourea

The methods described above may be used to prepare a large number of cytofectins for screening in a reasonable period of time. Synthesis in conjunction with screening can be used to efficiently select the most effective cytofectin for a defined application.

It is generally known to one skilled in the art that in the case of molecules having more than one reactive functional group, it is necessary to block or mask one or more of those groups which are not intended to participate in the particular coupling reaction.

#### Formulations

The compounds of the invention can be used in formulations to transfect mammalian cells both *in vitro* and *in vivo*. Formulations for transfection are known to those skilled in the art and are disclosed, together with methods for their preparation, for example, in U.S. Patent No. 5,264,618 to Felgner, U.S. Patent No. 5,334,761 to Gebeyehu et al, and Felgner et al. (*J. Biol. Chem.* 269:2550-2561, 1994), which are hereby incorporated by reference. The cationic lipids of the invention can be combined with amphipathic lipids such as phospholipids and with neutral lipids, such as cholesterol to form lipid vesicles, which can be liposomes, unilamellar vesicles, micelles, or simple films.

Cationic lipids of the invention are particularly useful in facilitating gene therapy, as disclosed in clinical trials reported by Nabel et al. (*Human Gene Therapy* 3:399-410, 1992). The use of cationic liposomes is known to be useful for facilitating entry of polynucleotides, macromolecules, and small molecules into cells of the blood vessels, the systemic circulation, lung epithelial cells, brain tissue and frog embryos (*Xenopus*).

It is also noted that the cytofectins of the present invention are useful in transfecting cells *in vitro*. Although various compounds within the scope of the present invention are somewhat tissue specific *in vivo*, most or all are useful for transfection of cultured cells *in vitro*. For any particular candidate cytofectin of the present invention, its relative transfection efficacy *in vitro* and in various tissues *in vivo* can be readily ascertained using screening assays such as those disclosed in Examples 8-12.

#### EXPERIMENTAL PROCEDURES

The chemical reactions described below are disclosed in terms of their general application to the preparation of the cationic lipids of the invention. Occasionally, the reaction may not be applicable as described to each molecular species within the disclosed scope. The compounds for which this occurs will be readily recognized by those skilled in the art. In all such cases, either the reactions can be successfully performed by conventional modifications known to those skilled in the art, that is, by changing to alternative conventional reagents, or by

routine modification of reaction conditions. Alternatively, other reactions disclosed herein or otherwise conventional will be applicable to the preparation of the compounds of the invention. In all preparative methods, all starting materials are known or readily preparable from known starting materials.

The present invention is described below in detail using the following examples, but the methods disclosed are applicable for the preparation of all cationic lipids covered by the scope of the invention and are not limited to the examples. All of the temperatures indicated in the Examples are in degrees Celsius and are uncorrected.

#### EXAMPLE 1

##### Preparation of a Carboxy cytofectin: DMRIE-Ox

DMRIE Br (1.14 g) is dissolved in 18 mL dimethylformamide with gentle stirring, then 2.71 g pyridinium dichromate is added and the reaction vessel is purged thoroughly with argon and tightly stoppered. After 18 h the solvent is removed at reduced pressure. The residue is partitioned between ethyl ether and 0.2N sodium hydroxide. The organic layer is collected and the aqueous layer is extracted repeatedly with ether. The combined organic extracts are dried with  $\text{MgSO}_4$ , then filtered and evaporated to afford crude product. Chromatography through silica gel with  $\text{CHCl}_3$ : $\text{MeOH}$ : $\text{NH}_4\text{OH}$ : $\text{H}_2\text{O}$  (90:10:0.25:0.25) affords pure product.

EXAMPLE 1A: DPRIE carboxylate; and EXAMPLE 1B: DORIE carboxylate were prepared following the procedure described in Example 1, using DPRIE Br and DORIE Br, respectively, as starting materials in place of DMRIE Br.

#### EXAMPLE 2

##### Preparation of a Carboxy cytofectin Ester: DMRIE-Ox Ethyl Ester

The carboxy cytofectin product of Example 1, DMRIE-Ox, (300 mg) was dissolved in 7 mL chloroform and washed with 3 mL 1N HCl. The organic phase was dried with  $\text{MgSO}_4$ , then filtered into a reaction vessel. Anhydrous ethanol (100  $\mu\text{L}$ ) and dicyclohexylcarbodiimide (1.3 mL of a 0.5 M solution in dichloromethane) were added, the flask was then stoppered and stirred overnight at ambient temperature. The reaction was filtered through a medium glass frit to remove any solid byproducts, then evaporated. Chromatography of the evaporation residue using silica gel with  $\text{CHCl}_3$ : $\text{MeOH}$ : $\text{NH}_4\text{OH}$ : $\text{H}_2\text{O}$  (90:10:0.25:0.25) afforded pure product.

#### EXAMPLE 3

##### Preparation of a Carboxy Cytofectin Amide: DMRIE-Ox Propyl Amide

DMRIE-Ox (300 mg) was dissolved in 7 mL chloroform and washed with 3 mL 1N HCl. The organic phase was dried with  $\text{MgSO}_4$ , then filtered into a reaction vessel. The solution was placed in an ice-water bath and N-hydroxysuccinimide (82 mg) then dicyclohexylcarbodiimide (1.3 mL of a 0.5 M solution in dichloromethane) were added and the reaction was allowed to stir with warming for 8 hours. At this time propyl amine (40 mg) was added as a neat liquid and the reaction allowed to stir at room temperature overnight. The reaction was filtered, then evaporated. Chromatography of the filtrate residue through silica gel with  $\text{CHCl}_3$ : $\text{MeOH}$ : $\text{NH}_4\text{OH}$ : $\text{H}_2\text{O}$  (90:10:0.25:0.25) afforded pure product.

EXAMPLE 3A: DMRIE carboxylate (methionine-methyl ester) amide; EXAMPLE 3B: DMRIE carboxylate (methionine-leucine-methyl ester) amide; and EXAMPLE 3C: DMRIE carboxylate (methionine-leucine-phenyl-alanine-methyl ester) amide were prepared in a similar procedure by substitution of the corresponding amines for propyl amine in the protocol of Example 3.

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## EXAMPLE 4

Preparation of a Cytoflectin Urea: DMRIE- $\beta$ -Methyl Urea

$\beta$ AE-DMRIE (1 g) was dissolved in 20 mL dry chloroform with stirring and methylisocyanate (100 mg) was added as a neat liquid. The reaction was stirred overnight then the solvent and excess isocyanate were removed by evaporation. The residue was chromatographed on silica gel with  $\text{CHCl}_3$ :MeOH:NH<sub>4</sub>OH:H<sub>2</sub>O which afforded pure product.

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EXAMPLE 4A:  $\gamma$ -aminopropyl-DMRIE, methylurea; and EXAMPLE 4B:  $\gamma$ -aminobutyl-DMRIE, methylurea were prepared similarly by substitution of  $\gamma$ -aminopropyl-DMRIE and  $\gamma$ -aminobutyl-DMRIE, respectively, as starting material.

## EXAMPLE 5

Preparation of a Cytoflectin Carbamate: DMRIE- $\beta$ -Methyl Carbamate

DMRIE (1 gm) was dissolved in 15 mL dry chloroform with stirring followed by addition of triethylamine (1.3 mL). Methylisocyanate (100 mg) was then added as a neat liquid, and the reaction was stirred overnight. The reaction was quenched by sequential addition of 20 mL chloroform and 20 mL 0.2N HCl. The biphasic mixture was stirred for approximately 1 h, then transferred to a separatory funnel and the organic phase collected. The solution was dried with magnesium sulfate, filtered, concentrated then treated with high vacuum to give the crude product as a foam. The material was chromatographed with silica gel using elution with  $\text{CHCl}_3$ :MeOH:NH<sub>4</sub>OH:H<sub>2</sub>O (90:10:0.25:0.25) to afford pure product.

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EXAMPLE 5A: hydroxypropyl DMRIE methyl carbamate; and

EXAMPLE 5B: hydroxybutyl DMRIE methyl carbamate were prepared similarly by substitution of hydroxypropyl DMRIE and hydroxybutyl DMRIE, respectively, as starting material.

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## EXAMPLE 6

General Scheme for Urea-linked Amino AcidsPreparation of DMRIE- $\gamma$ -U-Arg(NO<sub>2</sub>)-OMe:

A dry reaction vessel equipped with magnetic stirring was maintained under an argon atmosphere, then charged with 12 mL dry chloroform and 3.2 mL of 1.93 M phosgene in toluene. The flask was chilled in an ice/water bath and while stirring, a solution containing 1.40 gr  $\gamma$ AP DMRIE and 1.2 mL triethylamine in 12 mL dry chloroform was added dropwise over about 5 min. The reaction was stirred at ice/water temperature for 1 hr, then the cold bath removed and the reaction allowed to come to ambient temperature over 2.5 hr. At this time, a warm water bath was used to gently heat the reaction while excess reagents and solvent were removed evaporatively with a stream of dry argon over about 1 hr. The residue was redissolved in 20 mL chloroform and a solution containing 1.20 gr H-Arg(NO<sub>2</sub>)-OMe-HCL and 0.6 mL triethylamine in 7 mL dimethylformamide and 10 mL chloroform was added dropwise at ambient temperature with stirring over 5 min and the reaction allowed to stir overnight under an argon

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atmosphere. Evaporative removal of the solvent followed by chromatography on silica gel using a chloroform:methanol:aqueous ammonia solvent system afforded 1.47 gr of TLC homogenous product evidencing appropriate NMR, IR, UV and high resolution mass spectra.

EXAMPLE 6A:  $\gamma$ -aminopropyl-DMRIE, Lysine methyl ester urea; and EXAMPLE 6B:  $\gamma$ -aminopropyl-DMRIE, Lysine inner salt urea were prepared similarly by substituting DMRIE- $\gamma$ U-Lys(NO<sub>2</sub>)-OMe and DMRIE- $\gamma$ U-Lys(NO<sub>2</sub>), respectively, as starting material.

#### EXAMPLE 7

##### Intralung transfection assay

Adult (4-16 weeks) female BALB/c mice were lightly anesthetized with metophane and 132  $\mu$ g chloramphenicol acetyltransferase (CAT) DNA  $\pm$  cationic lipid in 100  $\mu$ l USP saline or water was delivered intranasally using a disposable sterile, plastic insulin syringe fitted with a small plastic catheter. All fluids and syringes were equilibrated to room temperature and the delivery of the single 100  $\mu$ l volume of DNA required less than one minute. Two or three days post-delivery, the mice were killed by sodium pentobarbital overdose, and the lungs extracted as follows.

Lungs were immediately frozen and stored at -78°C. Frozen lungs were individually pulverized into a fine powder by grinding over 0.4 ml frozen lysis buffer in a 1.5 ml tube using a reversible drill and a bit that just fits into the tube, and the powder is stored in the same tube at -78°C until extraction. Frozen powders are thawed and 100  $\mu$ l of Reporter Lysis Buffer from Promega (Catalog #E397A) is added to each. The samples were vortexed for 15 minutes, frozen-thawed three times using alternating liquid nitrogen and room temperature water baths and centrifuged three minutes at 10,000 x g. The supernatant was transferred to another 1.5 ml tube and the extraction process repeated (without freeze-thawing) after adding another 500  $\mu$ l lysis buffer to the pellet. The second supernatant was combined with the first and stored at -78°C.

The cationic lipids used were the DLRIE series (n=2-6) and the DOAP series wherein the alkyl chain has either 10, 12 or 14 carbon atoms. The DOAP series corresponds to formula 2 in which R1-R2-unbranched alkyl chain, n=1, R3-R4-CH<sub>3</sub>, m=3, G=N and R5=H.

CAT assays were performed by the radioactive partition method of Sankaran (*Anal. Biochem.*, 200:180-186, 1992) or by using a CAT ELISA kit (Boehringer Mannheim, Indianapolis, IN). Briefly, CAT tissue homogenates were disrupted by freeze-thawing three times in an ethanol/dry ice bath. Cellular debris was removed by centrifugation and the protein extract was incubated with <sup>14</sup>C-chloramphenicol and acetyl CoA. The chloramphenicol was extracted with ethyl acetate and thin layer chromatography was performed to determine the percent of <sup>14</sup>C-chloramphenicol converted by the extracted cellular protein. Cell extracts were standardized to 2  $\mu$ g protein incubated for 20 minutes. Tissue extracts were standardized to 200  $\mu$ g protein incubated for four hours.

Standard curves were constructed using purified enzyme (Sigma, St. Louis, MO) spiked into lung extracts or enzyme provided in the ELISA kit. The two CAT assay methods yielded equivalent pg CAT per sample from the same set of extracts. The results are summarized in Figures 2 and 3. For the DLRIE series, the most effective distance from the quaternary nitrogen at which to place the primary amine was four carbons (n=4). (Figure 2).



Figure 3 explores the effect of alkyl chain length on transfection efficiency for the DOAP series, indicating that the C12 compounds are significantly more effective at mediating pulmonary DNA transfection than are the C10 or C14 compounds. Figure 4 compares the activities of the amino acid linked compounds Gly-G-DLRIE and Lys-G-DLRIE relative to GXP-DLRIE. Gly-G-DLRIE was particularly effective in the murine lung system. Figure 4 demonstrates that linking an uncharged, non-polar amino acid such as glycine may to the cytofectin core structure may confer a higher level of transfection efficiency than that obtained with a charged, polar amino acid such as lysine.

The tests reported here not only indicate that the compounds of the present invention are active in transfection, but also demonstrate how to select and optimize cytofectins for transfection of particular tissues. Although particular optimum structures are readily apparent for this assay, it will be appreciated that these results are tissue specific; in other words, even cytofectins that performed suboptimally in this assay have valuable activity in other assays, such as *in vitro* transfection and intraperitoneal transfection.

#### EXAMPLE 8

##### Effect of Formulation on *in vitro* Transfection:

##### Comparison of GMU-DMRIE with DMRIE

**Cytofectin:** Solutions of a DMRIE or GMU-DMRIE in chloroform were prepared on a weight to volume (w/v) basis. Aliquots of cationic lipid and neutral lipid (when used) were transferred aseptically to sterile vials in amounts calculated to provide the relative and absolute lipid concentrations desired upon reconstitution with 1 ml of aqueous vehicle. Bulk chloroform was removed with a stream of dry nitrogen, and the vials were treated with high vacuum overnight to remove any residual solvent.

**DNA-lipid complexes:** Plasmid DNA at 5 mg/ml of phosphate buffered saline (PBS) as well as the dried, formulated cytofectin-neutral lipid mixture were suspended in OPTIMEM™ (Gibco BRL) and mixed together in 96 well plates at the desired mass/molar ratio as indicated in the Tables. The DNA-lipid complexes were added to the cells within 2 hours after mixing.

##### Transfection

**Cell Lines:** The cell lines used were obtained from the American Type Culture Collection (ATCC, Rockville, MD) as follows: COS7 monkey kidney cells (ATCC CRL 1651); and C2C12 mouse myoblast muscle cells (ATCC CRL 1772).

All cells were passaged 1:5 to 1:10 in 10% fetal bovine serum (FBS) and Dulbecco's Modified Eagles medium (DMEM). All cells were expanded through 10 doubling passages upon receipt and aliquots were stored frozen. Upon re-expansion, all cells were used for transfection studies before another 10 passages.

**Transfection Assays:** On day 0, 20,000 cells in 100 microliters 10% FBS/90% DMEM were seeded into each well of 96-well culture plates (Nunc) and cultured overnight in a 5% CO<sub>2</sub> incubator at 37°C. On Day 1, the medium was aspirated carefully without dislodging cells, and 100 microliters of GMU-DMRIE/pRSV *lacZ*/DOPE in serum-free OPTIMEM™ (Gibco BRL) was added. DMRIE was used as a reference standard. The *lacZ* gene encodes the enzyme  $\beta$ -galactosidase which can be assayed colorimetrically. The cationic lipid:DOPE ratios varied for each well. After 4 hours of culture, 50 microliters 30% FBS/70% OPTIMEM™ was added to each well. On Day 2, each

well received 100 microliters 10%FBS/90% OPTIMEM™. On Day 3, the medium was removed and 50 microliters lysis buffer (0.1% Triton-X100 in 250 mM Tris, pH 8.0) was added and the plates were stored at 70°C for at least 20 hours. After thawing, the well media were assayed for their content of  $\beta$ -galactosidase enzyme activity according to Felgner et al. (*J. Biol. Chem.* 269:2550-2561, 1994).

5 The results (Figures 1a-1d) show that total expression of  $\beta$ -gal in COS7 cells and C2C12 cells was optimal at a GMU-DMRIE/DOPE ratio of 50:50 in both cell lines. Additionally, in COS7 cells a GMU-DMRIE/DOPE ratio of 75:25 was also highly effective. Peak  $\beta$ -gal expression occurred at in COS7 cells at a GMU-DMRIE/DOPE ratio of 75:25 or 50:50. Similar ratios of GMU-DMRIE/DOPE gave peak  $\beta$ -gal activity in C2C12 cells. Total  $\beta$ -gal expression in both cell lines was significantly decreased when the ratio of GMU-DMRIE alone was used. GMU-DMRIE produced  
10 higher levels of activity than those achieved by DMRIE in COS7 cells. In C2C12 cells GMU-DMRIE gave significantly higher levels of total activity than DMRIE. The screening assay used in these tests is useful for demonstrating transfection activity and for optimizing the cytofectin/colipid ratio.

#### EXAMPLE 9

##### Intraperitoneal tumor assay

15 Two hundred thousand murine B16 tumor cells in 500  $\mu$ l RPMI were injected intraperitoneally into C57/B16 mice at day 0. At day 7-14, mice received intraperitoneal injections of 0.5 mg CAT DNA in 1.5 ml saline containing cytofectin at a 10:1 molar ratio of cytofectin:DNA. The cationic lipids used were DMRIE, GAP-DMRIE, GAP-DLRIE, and GMU-DLRIE. Two days later, tumors were collected, extracted and assayed for CAT activity as  
20 described in Example 7. The CAT activities observed with the cytofectin formulations is compared to a control receiving DNA alone in Figure 5. Each cationic lipid resulted in significant CAT expression in tumor cells, indicating entry and functional expression of the CAT DNA in the cells. CAT activity was greatest in cells transfected with DMRIE. GAP-DLRIE was nearly as effective as DMRIE while GAP-DLRIE and GAP-DMRIE also exhibited significant activity.

25 Figure 5 demonstrates that the cytofectins having a primary amine within three carbons of the quaternary nitrogen permit the efficient transfection of DNA in the intraperitoneal tumor assay.

#### EXAMPLE 10

##### Intramuscular assay

30 The quadriceps of restrained, awake mice are injected with 50  $\mu$ g luciferase or CAT DNA  $\pm$  cytofectin in 50  $\mu$ l USP saline using a disposable sterile, plastic insulin syringe fitted with a 28G 1/2 needle (Becton-Dickinson) and a plastic collar cut from a yellow Eppendorf micropipette tip. The collar length is adjusted to limit the needle orifice penetration to a distance of about 2 mm into the central part of the 3 mm diameter rectus femoris muscle. Injection fluids and syringes are equilibrated to room temperature and injection of the single 50  $\mu$ l volume of saline-DNA requires several seconds. The entire quadriceps muscle group (140-180 mg wet weight) is collected from each  
35 mouse leg at various times post-injection. Muscles are frozen and lysed as described in Example 7.

Luciferase activity is assayed using an automated microplate luminometer (Dynatech Model ML2250). One hundred  $\mu$ l of luciferase substrate is added by the luminometer's injection system to 20  $\mu$ l extract and sample light units are recorded. The luciferase content of the samples is calculated from Relative light Units using a standard curve of purified firefly luciferase performed in the presence of uninjected muscle extract. The luciferase activity present in the injected muscle extract is much higher than in the uninjected muscle extract.

This assay illustrates another screening assay for optimizing the structure of a particular cytofectin for use in a particular tissue.

#### EXAMPLE 11

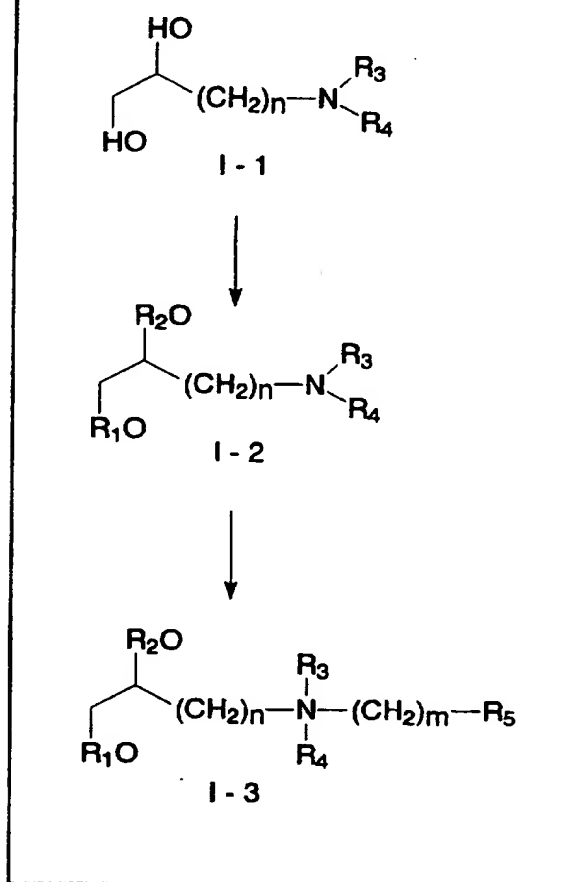
##### Gene transfer into porcine arteries and atherosclerotic rabbit arteries

Liposome transfection of porcine arteries is performed by anesthesia, intubation and sterile exposure of the iliofemoral arteries as described. (Nabel et al., *Science*, 249:1285-1288, 1990). A double balloon catheter is inserted into the iliofemoral artery, and the proximal balloon is inflated to 500 mm Hg for 5 minutes. The balloon is deflated and the catheter is advanced so that the central space between the proximal and distal balloon is irrigated with heparinized saline. The CAT DNA solution (CAT DNA  $\pm$  cytofectin) is instilled for 20 minutes in the central space of the catheter. The catheter is removed and antigrade blood flow is restored. Arteries are analyzed two days later for recombinant CAT expression. Arteries transfected with CAT DNA in the presence of cationic lipid exhibit a significant increase in CAT gene expression compared to arteries contacted with the DNA alone.

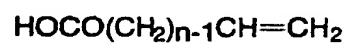
*In vivo* gene transfer of atherosclerotic rabbit iliac arteries is performed using a double injury model which is described by Faxon et al. (*Arteriosclerosis*, 4:189-195, 1984). After the second angioplasty injury is completed, the angioplasty balloon is withdrawn slightly so that the end hold infusion port of the catheter is at the proximal end of the injury. A ligature is placed at the distal end of the injury and the injured segment is flushed with heparinized saline and CAT DNA  $\pm$  cationic lipid liposome solution is instilled for 20 minutes into the isolated injured segment. The catheter is removed and antigrade blood flow is restored. Arteries are analyzed two days later for recombinant CAT expression. Arteries transfected with CAT DNA in the presence of cationic lipid exhibit a significant increase in CAT gene expression compared to arteries contacted with the DNA alone.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the invention to its fullest extent. The invention may be embodied in other specific forms without departing from its spirit of essential characteristics. The described embodiments are to be considered in all respects only as illustrative and not restrictive, and the scope of the invention is therefore indicated by the appended claims rather than by the foregoing description. All modifications which come within the meaning and range of the lawful equivalency of the claims are to be embraced within their scope.

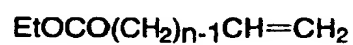
Schem I



## Scheme II



II - 1



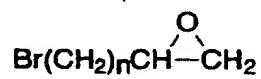
II - 2



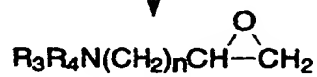
II - 3



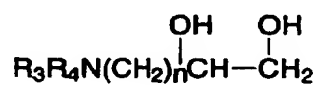
II - 4



II - 5

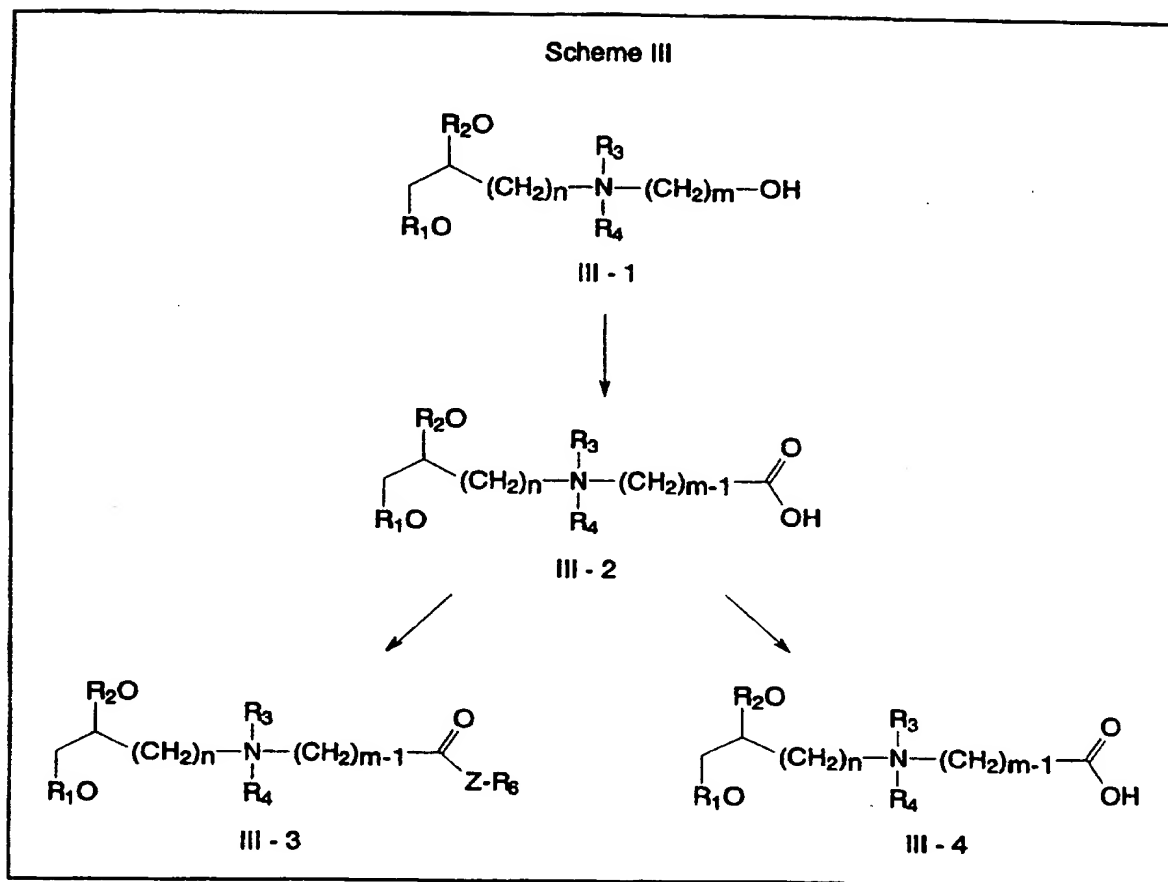


II - 6



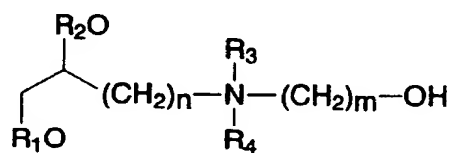
II - 7

Scheme III

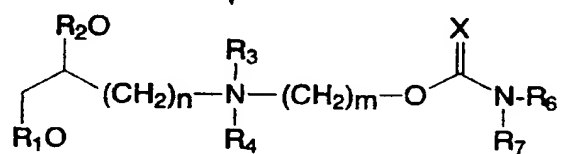


## Schem IV

## Orientation A

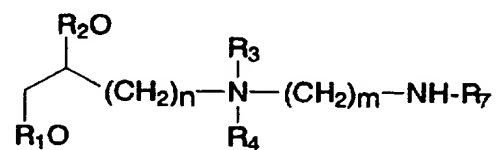


IV A - 1

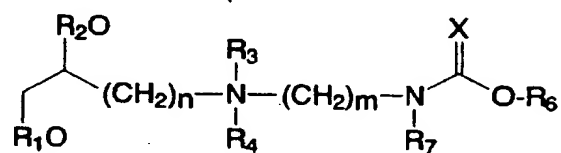


IV A - 2

## Orientation B

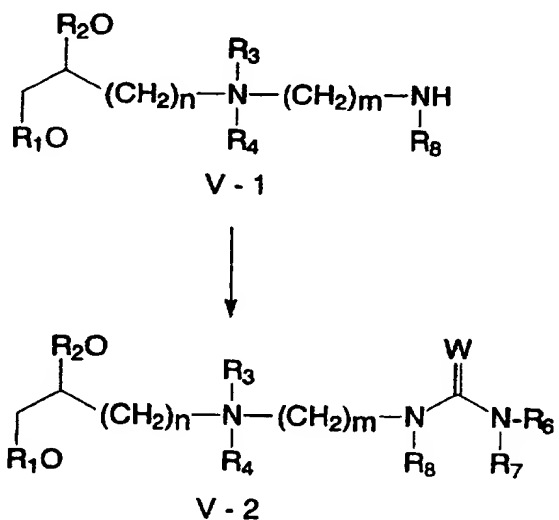


IV B - 1



IV B - 2

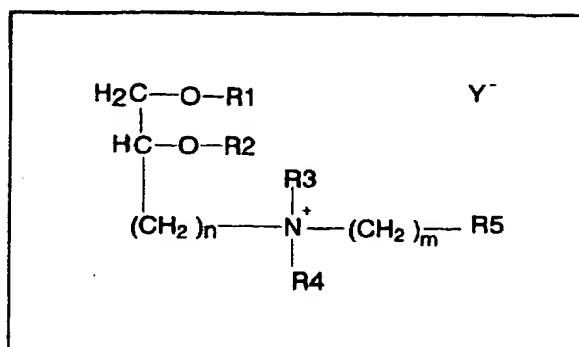
## Scheme V





## WHAT IS CLAIMED IS:

1. A cationic amphiphilic lipid comprising:
  - a Rosenthal inhibitor core structure comprising a quaternary nitrogen group;
  - an alkyl linker attached to said quaternary nitrogen, said linker comprising a carboxy, carbamyl, ureyl, thioureyll or guanidyl group; and
  - a bioactive agent which is recognized by a metabolic sytem attached to said linker.
2. A compound of the formula

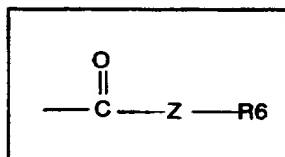


wherein

R1 and R2 are independently H, linear or branched, unsubstituted or substituted  $\text{C}_{1-23}$  alkyl, acyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-\text{O}(\text{CH}_2)_k-\text{CH}_3$ ,  $-\text{S}(\text{CH}_2)_k-\text{CH}_3$ ,  $\text{X}(\text{CH}_2)_k-$ , wherein X is a halide, and  $-\text{N}((\text{CH}_2)_k-\text{CH}_3)_2$ , wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0 to 4.

R3 and R4 are independently linear or branched, unsubstituted or substituted  $\text{C}_{1-23}$  alkyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-\text{O}(\text{CH}_2)_k-\text{CH}_3$ ,  $-\text{S}(\text{CH}_2)_k-\text{CH}_3$ ,  $\text{X}(\text{CH}_2)_k-$ , wherein X is a halide, and  $-\text{N}((\text{CH}_2)_k-\text{CH}_3)_2$ , wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0-4;

R5 has the structure



wherein Z is selected from the group consisting of O, S, NH, Se, C, H and OH; and

R6 is absent, or is selected from the group defined for R1, R2, R3 and R4;

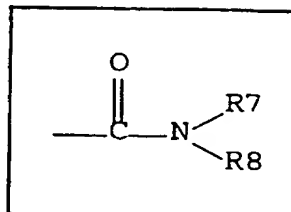
n is 1 to 6;

m is 2 to 10; and

Y is a pharmaceutically acceptable anion.

3. A compound according to Claim 2 wherein

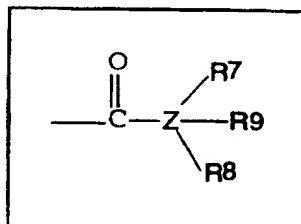
Z is N, and R5 has the structure



wherein

R7 and R8 are independently selected from the group defined for R1, R2, R3 and R4 and optionally further comprise a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono-, di- or polysaccharide, or other bioactive or pharmaceutical agent.

4. A compound according to Claim 2 wherein Z is C, and R5 has the structure

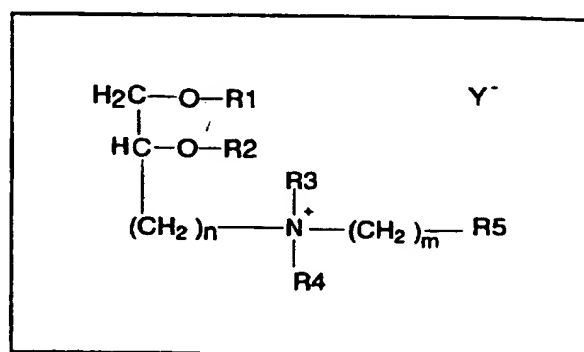


wherein

R7, R8 and R9 are independently selected from the group defined for R1, R2, R3 and R4 and optionally further comprise a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono-di- or polysaccharide, or other bioactive or pharmaceutical agent.

5. A compound according to Claim 2 wherein R1 and R2 are C<sub>10</sub> to C<sub>20</sub> alkyl or alkenyl groups, Z is O and R6 is an amino acid or peptide linked to Z as an ester.

6. A compound of the formula

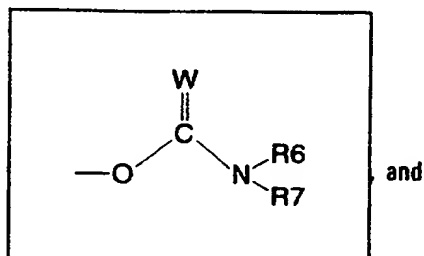
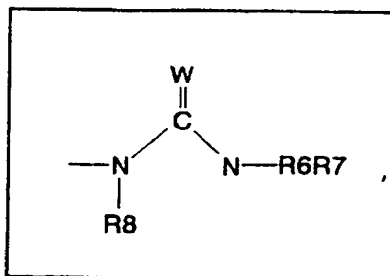


wherein

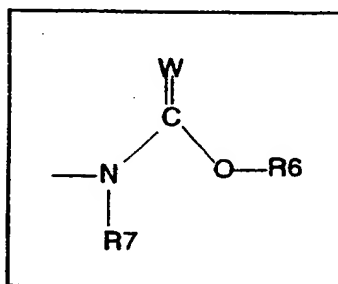
R1 and R2 are independently H, linear or branched, unsubstituted or substituted  $C_{1-23}$  alkyl, acyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0-4;

R3 and R4 are independently linear or branched, unsubstituted or substituted  $C_{1-23}$  alkyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from  $-O-(CH_2)_k-CH_3$ ,  $-S-(CH_2)_k-CH_3$ ,  $X-(CH_2)_k-$ , wherein X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0-4;

wherein R5 is  $N-C(O)-$  or is selected from the group consisting of



and



wherein

R6, or R6 together with R7, is selected from the group

defined for R1, R2, R3 and R4 and optionally further

comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent;

R8 is absent, or is H or an alkyl group selected from the group consisting of R1, R2, R3 and R4 and wherein R8 may be joined to R6 or R7 so as to form a ring;

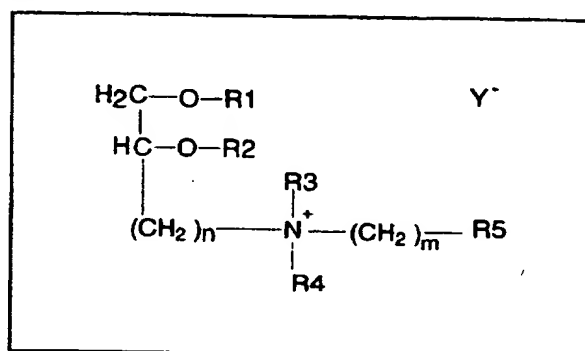
W is O, N, NH, S, Se, C, CH, or CR<sub>1</sub>R<sub>2</sub>, wherein R<sub>1</sub> and R<sub>2</sub> are as defined above;

n is 1 to 6;

m is 2 to 10; and

Y is a pharmaceutically acceptable anion.

7. A compound of the formula



wherein

R1 and R2 are independently H, linear or branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, acyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms k is 0-4, n is 1-6, and m is 2-10; and

R3 and R4 are independently linear or branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0-4;

wherein R5 is H or is -O-R6-R7, wherein R6, or R6 together with R7, is selected from the group defined for R1, R2, R3 and R4 and optionally further comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent;

n is 1 to 6;

m is 2 to 10; and

Y is a pharmaceutically acceptable anion;

wherein if R1 is H, or C1-C24 alkyl or alkenyl and R2 is C1 to C24 alkyl or alkenyl and R3 and R4 are the same or different and are C1-C24 alkyl or H, and R5 is O-R6-R7, and R6 is absent, -C(O)-(CH<sub>2</sub>)<sub>p</sub>-NH, a diaminocarboxylate ester group which is alkyl, aryl, or aralkyl or -C(O)-(CH<sub>2</sub>)<sub>p</sub>-NH linked to a diaminocarboxylate ester group and p is 1 to 18, then R7 is not H, spermine, spermidine, a histone, a protein with DNA-binding specificity or the preceding groups wherein the amine functionalities of the R7 moiety are quaternized with H or a C<sub>1</sub> to C<sub>24</sub> straight or branched alkyl chain; or

an L- or D-α amino acid having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or analogues thereof; or

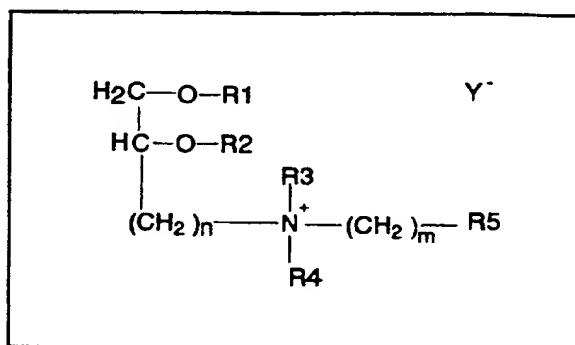
wherein the amine of the R7 moiety is quaternized with H or a C<sub>1</sub> to C<sub>24</sub> straight or branched alkyl chain; or

a polypeptide selected from the group consisting of L- or D-α amino acids, wherein at least one of the amino acid residues comprises arginine, histidine, lysine, ornithine, or analogues thereof.

8. A compound according to Claim 7 selected from the group consisting of amino acids or peptides chemically linked to dialkyl-Rosenthal inhibitor ethers or dialkenyl-Rosenthal inhibitor ethers wherein said amino acids or peptides are selected from the group consisting of those groups which are non-polar, polar and uncharged, or negatively charged at physiological pH.

9. A compound according to Claim 7 selected from the group consisting of dialkyl- and dialkenyl-Rosenthal Inhibitor ethers covalently linked to a bioactive moiety through a bifunctional linker.

10. A compound of the formula



wherein

R1 and R2 are independently H, linear or branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, acyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0-4.

R3 and R4 are independently linear or branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein

X is a halide, and  $-N((CH_2)_k-CH_3)_2$ , wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0-4;

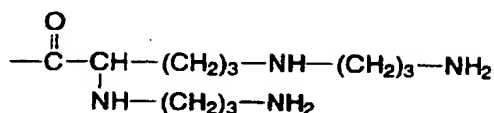
wherein R5 is H or NH-R6-R7, wherein R6, or R6 together with R7, is selected from the group defined for R1, R2, R3 and R4 and optionally further comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent;

n is 1-6;

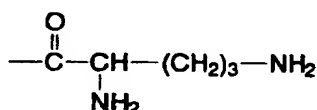
m is 1-10; and

Y is a pharmaceutically acceptable anion; and

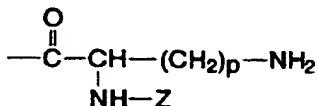
wherein if n is 1, and m is 2 to 6, and R1 and R2 separately or together are  $C_1-C_{23}$  alkyl or  $C(=O)-C_1-C_{23}$ , and R3 and R4 separately or together are H or unbranched alkyl  $C_1-C_6$ , and R5 is NH-R6-R7 then R6-R7 is not  $(CH_2)_zNH_2$  where z is 2-6; or  $-(CH_2)_3-NH-(CH_2)_4-NH_2$ ; or  $-NH-(CH_2)_3-NH-(CH_2)_4-NH(CH_2)_3-NH_2$ , C(=O)-fluorescein, or



or



or



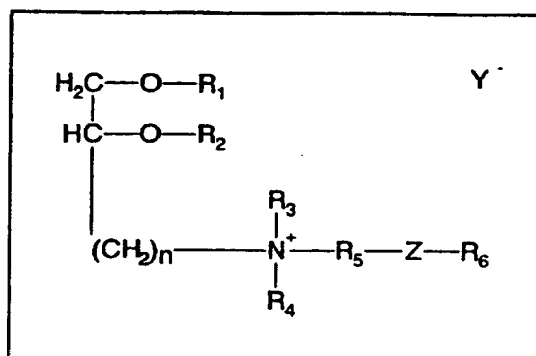
where p is 2-5, Z is H or other groups attached by amide or alkyl amino groups.

11. A compound according to Claim 10 selected from the group consisting of amino acids or peptides chemically linked to dialkyl-Rosenthal inhibitor ethers or dialkenyl-Rosenthal inhibitor ethers wherein said amino acids or peptides are selected from the group consisting of those groups which are non-polar, polar and uncharged, and negatively charged at physiological pH.

12. A compound according to Claim 10 selected from the group consisting of dialkyl- and dialkenyl-Rosenthal Inhibitor ethers covalently linked to a bioactive moiety through a bifunctional linker.

13. The compound of Claim 10 wherein R5 is glycine.

14. A compound having the structure



15 wherein R1 and R2 are independently H, linear or branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, acyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0-4;

20 R3 and R4 are independently linear or branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, alkylene or heteroalkyl groups having from 0 to 6 sites of unsaturation, cyclic and aryl groups, said groups comprising from 0 to 5 heteroatoms, wherein the substituent groups are selected from -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0-4;

R5 is selected from the group defined for R1-R5;

25 wherein R6, or R6 together with R7, is selected from the group defined for R1, R2, R3 and R4 and optionally further comprises a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono, di- or polysaccharide, or other bioactive or pharmaceutical agent; and

Z is O, NH, or S;

n is 1 to 6;

30 m is 2 to 10; and

Y is a pharmaceutically acceptable anion;

35 wherein if Z is O and R1 is H, or C1-C24 alkyl or alkenyl and R2 is C1 to C24 alkyl or alkenyl and R3 and R4 are the same or different and are C1-C24 alkyl or H, and R6 is absent, -C(O)-(CH<sub>2</sub>)<sub>p</sub>-NH, a diaminocarboxylate ester group which is alkyl, aryl, or aralkyl or -C(O)-(CH<sub>2</sub>)<sub>p</sub>-NH linked to a diaminocarboxylate ester group and p is 1 to 18, then R7 is not H, spermine, spermidine, a histone, a protein with DNA-binding specificity or the preceding

groups wherein the amine functionalities of the R7 moiety are quaternized with H or a C<sub>1</sub> to C<sub>24</sub> straight or branched alkyl chain; or

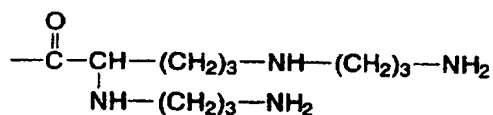
an L- or D- $\alpha$  amino acid having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or analogues thereof; or

5 wherein the amine of the R7 moiety is quaternized with H or a C<sub>1</sub> to C<sub>24</sub> straight or branched alkyl chain; or

a polypeptide selected from the group consisting of L- or D- $\alpha$  amino acids, wherein at least one of the amino acid residues comprises arginine, histidine, lysine, ornithine, or analogues thereof; and

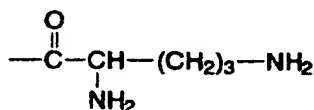
10 wherein if Z is NH and n is 1 and m is 2 to 6, and R1 and R2 separately or together are C<sub>1</sub>-C<sub>23</sub> alkyl or C(O)-C<sub>1</sub>-C<sub>23</sub>, and R3 and R4 separately or together are H or unbranched alkyl C<sub>1</sub>-C<sub>6</sub>, and R5 is NH-R6-R7 then R6-R7 is not -(CH<sub>2</sub>)<sub>z</sub>NH<sub>2</sub> where z is 2-6; or -(CH<sub>2</sub>)<sub>3</sub>-NH-(CH<sub>2</sub>)<sub>4</sub>-NH<sub>2</sub>; or -NH-(CH<sub>2</sub>)<sub>3</sub>-NH-(CH<sub>2</sub>)<sub>4</sub>-NH(CH<sub>2</sub>)<sub>3</sub>-NH<sub>2</sub>, C(O)-fluorescein, or

15



or

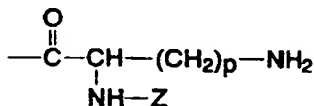
20



25

or

30



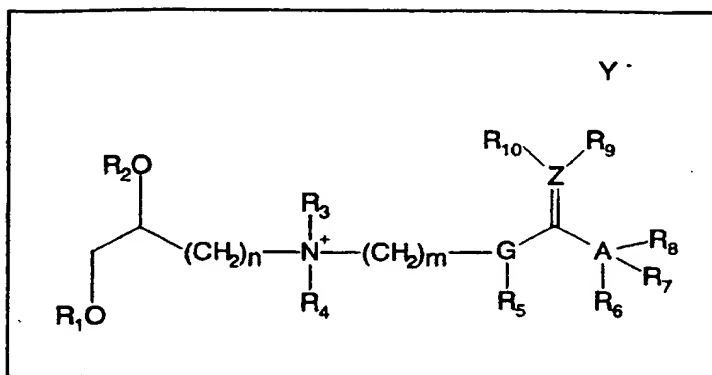
where p is 2-5, Z is H or other groups attached by amide or alkyl amino groups.

15. A compound according to Claim 14 wherein R5 is a heteroethylene group, Z is O and R6 together with R7 is a amino acid or peptide chemically linked to the heteroethylene group.

35

16. A compound having the structure:





wherein

10 R1 and R2 are independently H, linear, branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, acyl, alkylene, or heteroalkyl groups having from 0 to 6 sites of unsaturation, or cyclic or aryl groups, said cyclic or aryl groups containing up to five heteroatoms, wherein the substituent groups are selected from the group consisting of -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein k is 0 to 4, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms;

15 R3 and R4 are independently linear, branched, unsubstituted or substituted C<sub>1-23</sub> alkyl, acyl, alkene, or heteroalkyl groups having from 0 to 6 sites of unsaturation, or cyclic or aryl groups, said cyclic or aryl groups containing up to five heteroatoms, wherein the substituent groups are selected from the group consisting of -O-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, -S-(CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>, X-(CH<sub>2</sub>)<sub>k</sub>-, wherein k is 0 to 4, wherein X is a halide, and -N((CH<sub>2</sub>)<sub>k</sub>-CH<sub>3</sub>)<sub>2</sub>, wherein the alkyl groups of said substituents comprise from 0 to 2 heteroatoms and k is 0 to 4;

20 R5 is absent, H or an alkyl group as defined for R<sub>1</sub> and R<sub>2</sub>; R5 through R10 independently or in combination are absent, or are H or alkyl groups as defined for R<sub>1</sub> and R<sub>2</sub> and, optionally, further comprise a chemically linked amino acid, peptide, polypeptide, protein, nucleic acid, nucleotide, polynucleotide, mono-, di- or polysaccharide, or other bioactive or pharmaceutical agent;

G is absent, O, N, S or Se;

25 Z is O, N, S, Se, C;

A is O, N, S, Se, C;

n is 1 to 6;

m is 2-10;

Y is a pharmaceutically acceptable anion;

30 wherein if G is N and Z is O, then A is not C;

wherein if G is O and Z is O then A is not C;

and wherein if G is C and Z is C then A is not C.

17. The compound of Claim 16 having a primary amine within 8 atoms of the quaternary nitrogen.

18. The compound of Claim 16 wherein if any of R5-R10 are amino acids or peptides they are selected from the group consisting of those amino acids and peptides which are non-polar, polar and uncharged, and negatively charged at physiological pH.

35

19. The compound of claim 16 wherein if any of R5-R10 are amino acids or peptides they comprise at least one amino acid not generally found in natural organisms.

20. Cytofectin formulations comprising the compounds of Claims 16 in a physiologically or isotonic acceptable solution.

5 21. Cytofectin formulations comprising the cationic lipids of Claim 16 and a co-lipid selected from the group consisting of neutral lipids, phospholipids, and cholesterol in a suitable carrier solution.

22. A method of delivering an anionic molecule into a cell comprising the steps of  
(a) contacting the anionic molecule with a formulation comprising an effective amount of any of the cationic lipids of Claim 16 to form a complex with the lipid; and

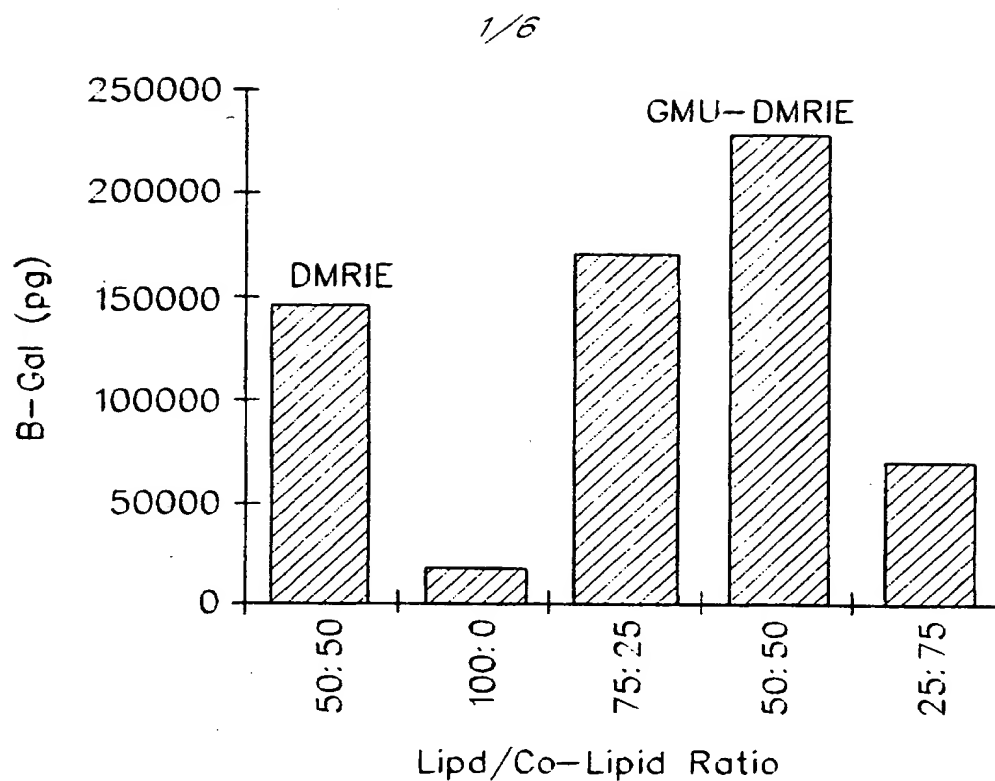
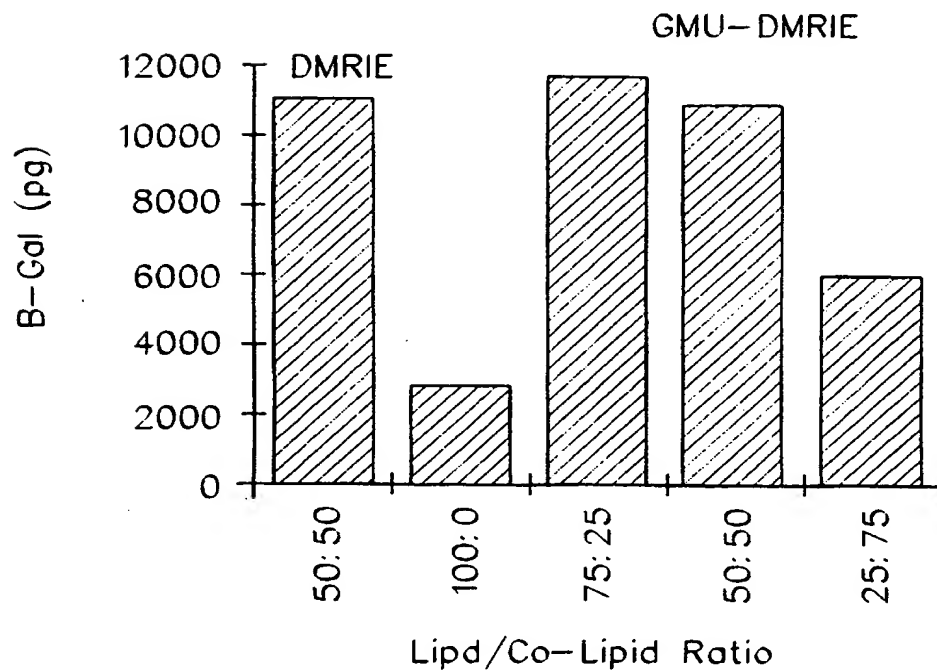
10 (b) contacting a cell with the lipid complex formed in step (a);  
whereby a biologically effective amount of the anionic molecules are inserted into the cell.

23. Use of the compound of Claim 16 in a composition for introducing a bioactive agent into a cell.

24. The use of Claim 23, wherein the cell is *in vitro*.

25. The use of Claim 23, wherein the cell is *in vivo*.

15 26. The use of Claim 23, wherein the bioactive agent is anionic.

*FIG. 1A**FIG. 1B*

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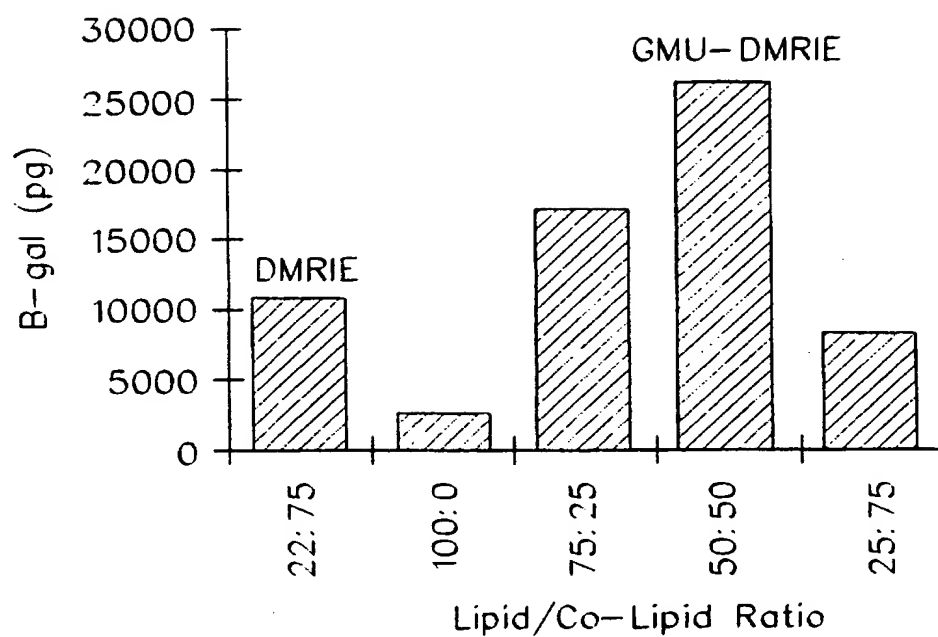


FIG. 1C

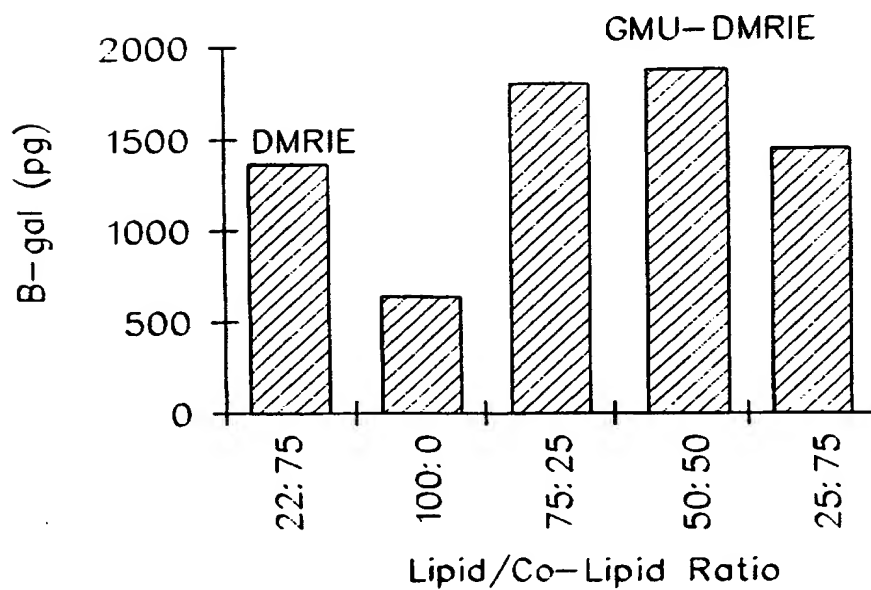
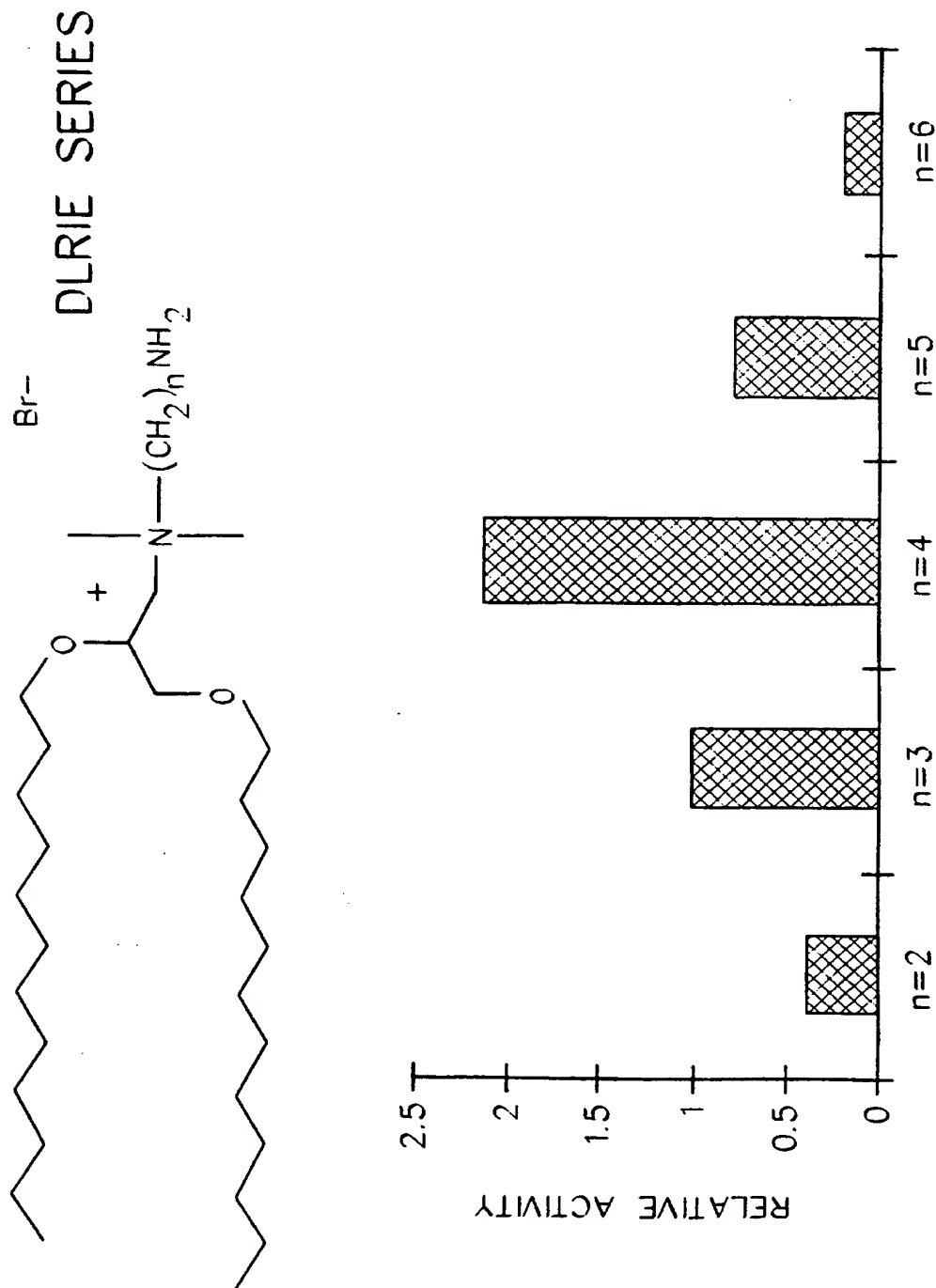


FIG. 1D

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FIG. 2

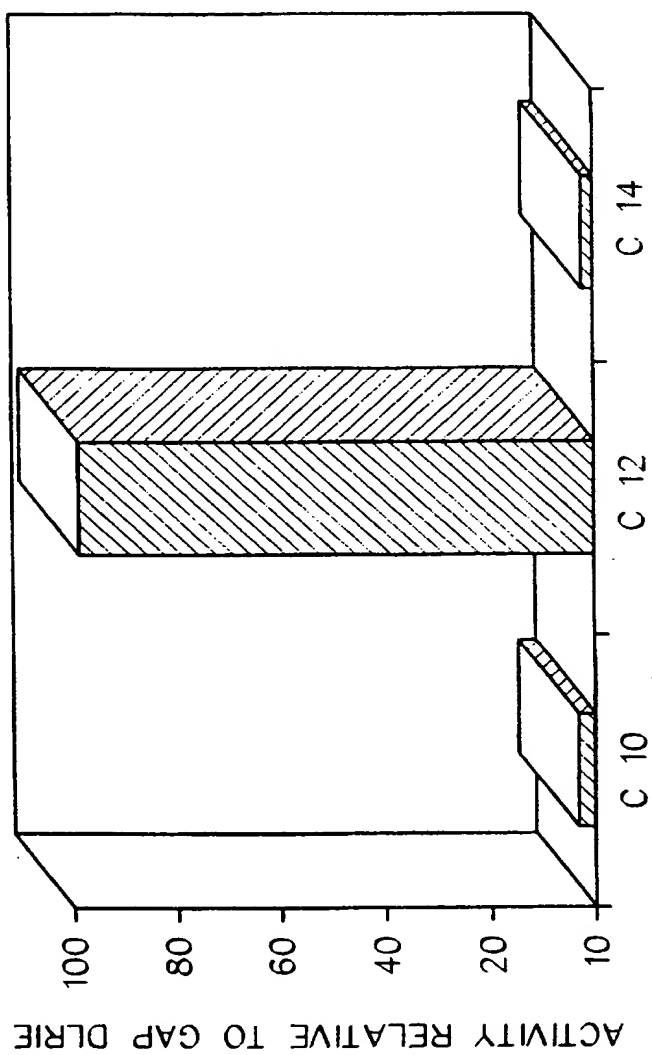
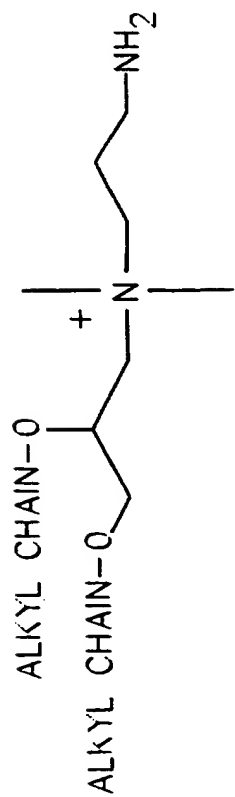


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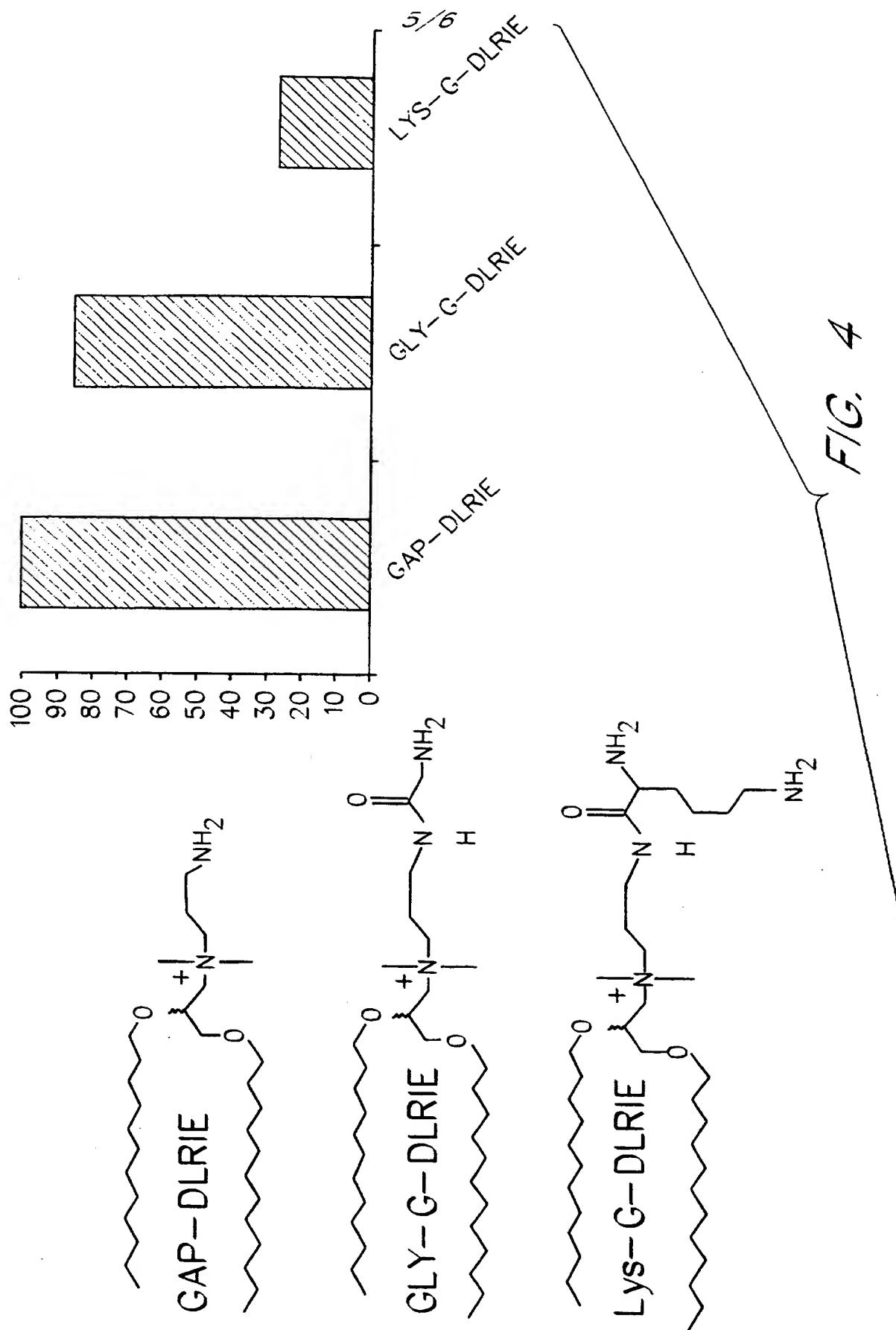
4/6

FIG. 3

## DOAP SERIES

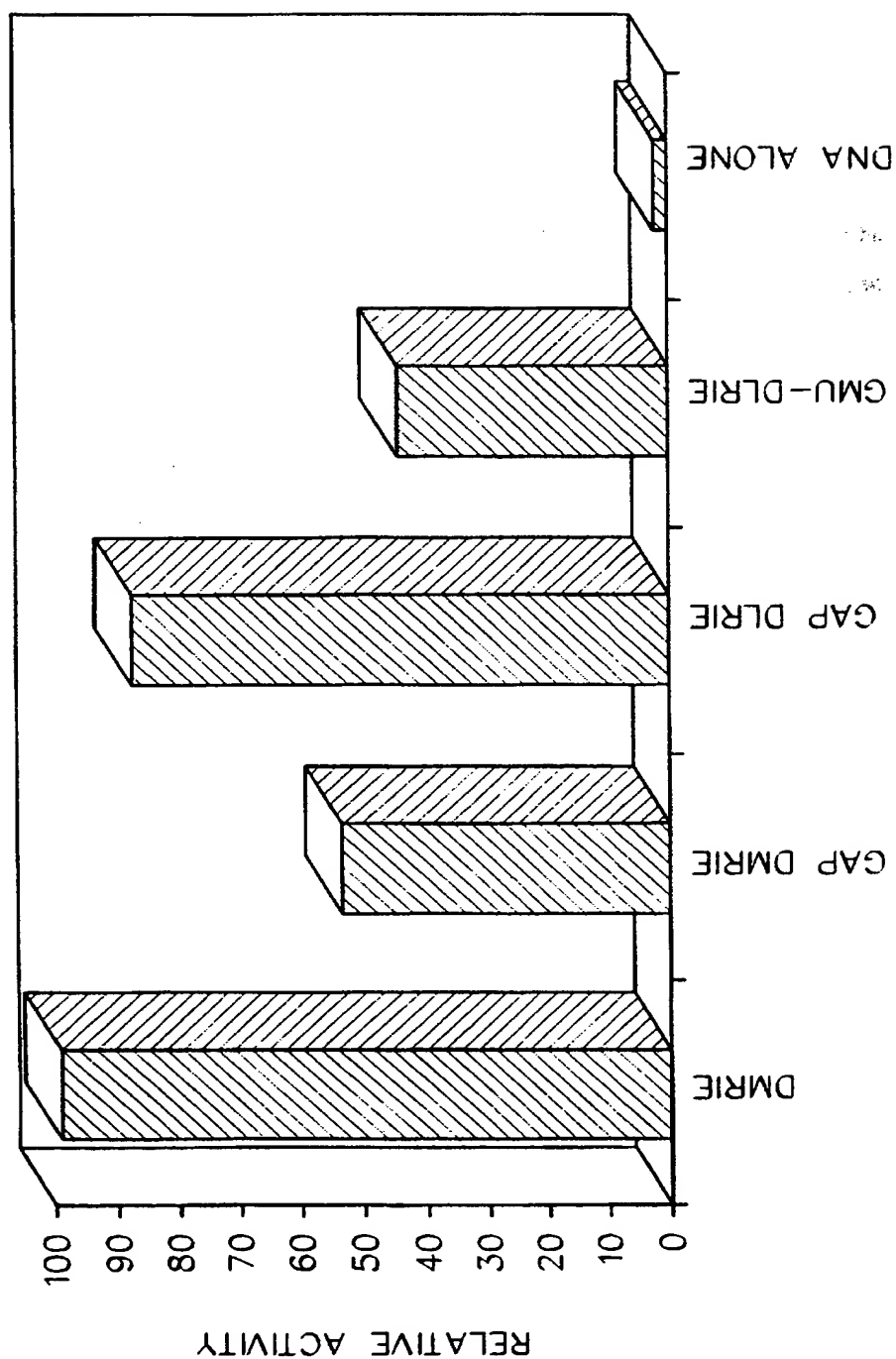


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CONDITIONS: 0.5mg DNA, 10/1 mol/mol DNA/Cctofectin in 1.5 mL saline

FIG. 5





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 9/127, C07C 229/12, 237/06, 279/12, 275/16, 271/20</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 97/19675</b> <b>(43) International Publication Date:</b> 5 June 1997 (05.06.97)
<b>(21) International Application Number:</b> PCT/US96/19721 <b>(22) International Filing Date:</b> 27 November 1996 (27.11.96)  <b>(30) Priority Data:</b> 08/565,756 30 November 1995 (30.11.95) US  <b>(71) Applicant:</b> VICAL INCORPORATED [US/US]; Suite 100, 9373 Towne Centre Drive, San Diego, CA 92121 (US). <b>(72) Inventor:</b> WHEELER, Carl, J.; 14101 Arbolitos Drive, Poway, CA 92064-6439 (US). <b>(74) Agent:</b> ALTMAN, Daniel, E.; Knobbe, Martens, Olson & Bear, 16th floor, 620 Newport Center Drive, Newport Beach, CA 92660 (US).		<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 2 October 1997 (02.10.97)
<b>(54) Title:</b> COMPLEX CATIONIC LIPIDS  <b>(57) Abstract</b>  Cationic lipids, having a derivated quaternary ammonium head group, that provide improved cell targeting ability and enhanced transfective efficacy for negatively charged macromolecules. The lipids comprise a linker having functional groups that provide sites for further attachment of drugs, cell receptor ligands or other bioactive agents.		

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# INTERNATIONAL SEARCH REPORT

Internat. Application No  
PCT/US 96/19721

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K9/127 C07C229/12 C07C237/06 C07C279/12 C07C275/16  
C07C271/20

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 17424 A (VICAL INC) 14 November 1991 see abstract; claims 12,18,39 ---	1,2
X	US 5 264 618 A (FELGNER PHILIP L ET AL) 23 November 1993 cited in the application see claims 1-12 ---	1,2
X	PATENT ABSTRACTS OF JAPAN vol. 016, no. 063 (C-0911), 18 February 1992 & JP 03 258900 A (KAO CORP), 19 November 1991, see abstract ---	2
E	EP 0 747 351 A (GEN PROBE INC) 11 December 1996 see page 5 --- -/-	1,2

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- \*&\* document member of the same patent family

Date of the actual completion of the international search

10 April 1997

Date of mailing of the international search report

21.08.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
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Fax: (+31-70) 340-3016

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# INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>FELGNER J H ET AL: "ENHANCED GENE DELIVERY AND MECHANISM STUDIES WITH A NOVEL SERIES OF CATIONIC LIPID FORMULATIONS"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 4, 28 January 1994, pages 2550-2561, XP002010485</p> <p>cited in the application</p> <p>see the whole document</p> <p>-----</p>	1

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 19721

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 3-4  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
  
The subject-matter of the claims 3 and 4 is unclear, since the definition of R5 is unclear.
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See continuation sheet (PCT/ISA/210)

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,2,5,6,16-26 (partly)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/ 19721

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

- 1) Claims: 1,2 (partly), 5,6,16-26 (partly):  
A cationic amphiphilic lipid comprising a Rosenthal inhibitor core structure comprising a quaternary nitrogen group, a linker, and a bioactive agent, characterised by the fact that the linker contains a carboxyl, carbamyl, ureyl, thioureyll or guanidyl group (common feature)
2. Claims: 2 (partly):  
Compounds according to the generic formula of claim 2 without the common feature and with a group  $R5 = -C(O)-Z-R6$
3. Claims: 7-9, 10 (partly), 14 (partly):  
Compounds according to the generic formula without the common feature and with  $R5 = \text{Hydrogen or } -OR6R7$
4. Claims: 10 (partly) - 13, 14 (partly):  
Compounds according to the generic formula without the common feature and with  $R5 = \text{Hydrogen or } -OR6R7$
5. Claims: 14 (partly), 15:  
Compounds of the generic formula of claim 14 without the common feature.
6. Claims: 16-26 (partly):  
Compounds of the generic formula of claim 16 without the common feature.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/19721

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9117424 A	14-11-91	AU 7979491 A	27-11-91
US 5264618 A	23-11-93	AU 7854791 A	11-11-91
		EP 0523189 A	20-01-93
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EP 0747351 A	11-12-96	AU 5862296 A	30-12-96
		WO 9640627 A	19-12-96

